

Where theology matters

The voices of religion are more prominent and influential than they have been for many decades. Researchers, religious and otherwise, need to come to terms with this, while noting that some dogma is not backed by all theologians.

Theologians and philosophers have been arguing about religion and science for centuries, so we won't presume to break any new ground here. Besides, nearly 800 years ago, Thomas Aquinas found a way to reconcile the two — as did Einstein, who wrote in 1930 that: "The cosmic religious feeling is the strongest and noblest motive for scientific research." Why not just leave it at that? Why not simply accept Pope John Paul II's view that science and religion each "bring out different aspects of reality"?

The reason is that the two traditions regularly stray onto each other's territories and stir up trouble. Consider the political battles over the teaching of 'creationism' and 'intelligent design' in schools — an attempt by some religious people to foist their beliefs, masquerading as science, on others. Science bases its conclusions on empirical data, not on the authority of the Talmud, Bible or Koran. And even though some may find it distressing that science recognizes no god, forcing it to do so will only produce bad science.

Meanwhile science, allied with business, is encroaching on religion's turf by unleashing technologies that raise profound questions about human nature. Religious thinkers and secular ethicists are right to raise concerns, and scientists shouldn't just charge ahead without listening to them. Perhaps researchers will find a way to extract stem cells without destroying the early embryo, and today's hot bioethical dilemma will go away. But others will come along to replace it.

Jewish bioethicist and chairman of the US President's Council on Bioethics, Leon Kass, cuts to the heart of the problem: "Victory over mortality is the unstated but implicit goal of modern medical science." And immortality has long been the realm of religion.

Medical science aims to relieve suffering — an unquestionably noble goal. But religion thinks it wrong to emphasize this value over all

others. Kass again: "In parallel with medical progress, a new moral sensibility has developed that serves precisely medicine's crusade against mortality: anything is permitted if it saves life, cures disease or prevents death." Most religions accept the inevitability of suffering and death, and seek to invest life with meaning, rather than simply extend it.

Religion and science also have different methods and standards. "Proponents of human embryo research have argued that all scientifically sound lines of research should be pursued simultaneously," writes Kevin FitzGerald, a Jesuit priest and molecular biologist at Georgetown University. "From a scientific perspective, this approach makes the most sense. In science, when there is uncertainty, one does all the research indicated to gain the desired knowledge and understanding," he adds. "But what is best for science is not always best for a society and its members."

FitzGerald would avoid experiments he finds ethically unacceptable. Others ask why society should be denied a medical advance just because some of its members find it morally troubling. This is a real concern if, as suggested on page 666, those morals are expressed with more conviction that some theologians would support. For example, not all Catholics believe that the Vatican's position on the status of embryos rules out embryonic stem-cell research.

Too often the debate is full of the rhetoric of televangelists and biotech lobbyists, who caricature each other as godless Frankensteins and ignorant Bible-thumpers. But looking back through testimony put before the President's Council on Bioethics, one is struck by the high-mindedness and sincerity of the discussion.

Secular scientists (probably the majority) should avoid underestimating the influence and rights of those who believe that only a god can give meaning to the world, human suffering and mortality. ■

Spinning out of control

Researchers should beware of 'public relations' screens that are anything but helpful to science communication.

Those who report on science should give constant thanks to those scientists who explain their work with generosity and patience. Cynics who argue that researchers just crave publicity should recognize that the enthusiasm with which such information is typically imparted belies any suspicion of self-serving motives.

Paradoxically, this willingness to engage with journalists is threatened by the idea in the scientific community of 'public engagement'. Many companies and research institutes now have slick PR offices in which the ethos is informed not by the scientific tradition of exchange and interaction, but by a culture of marketing. It may be natural that organizations want to trumpet their achievements in triumphant press releases, and journalists are generally canny enough to decode the hype. And it is unsurprising that large companies often demand that their researchers be chaperoned in interviews by press officers.

It is more disturbing when government-funded research agencies, such as the US National Institutes of Health, erect PR screens between their scientists and the media, so that all correspondence is mediated via e-mail by a press officer. One result is that scientists

might come to believe it sufficient to respond to enquiries about publicly funded research with chunks of management jargon.

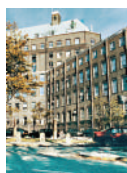
A recent set of questions from a journalist was sifted by this mechanism to elicit the following response, doctored here to spare some blushes: "Based on past and current progress, the NIH believes that mouthwashology is a key enabling technology platform with the potential to transform the diagnosis, treatment and prevention of halitosis. NIH's recently announced Alliance for Halitosis is designed to facilitate and accelerate the progress of the twenty-first century research teams needed to realize the promise of these and future mouthwash technologies for halitosis sufferers." A polite follow-up message from the reporter, tactfully refraining from pointing out that this statement was useless to any self-respecting journalist but suggesting that it failed to address any of the questions originally posed, met with stony silence.

It will be a sad day if scientists start to believe that this sort of bland and meaningless corporate-speak absolves them of the responsibility to tell people what they are actually doing. ■





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Curators bugged by museum's vision for insect collection

Jim Giles, London

Plans to move one of the world's premier natural history collections into new state-of-the-art premises are being resisted by researchers, who say that the relocation will hurt their ability to do their work.

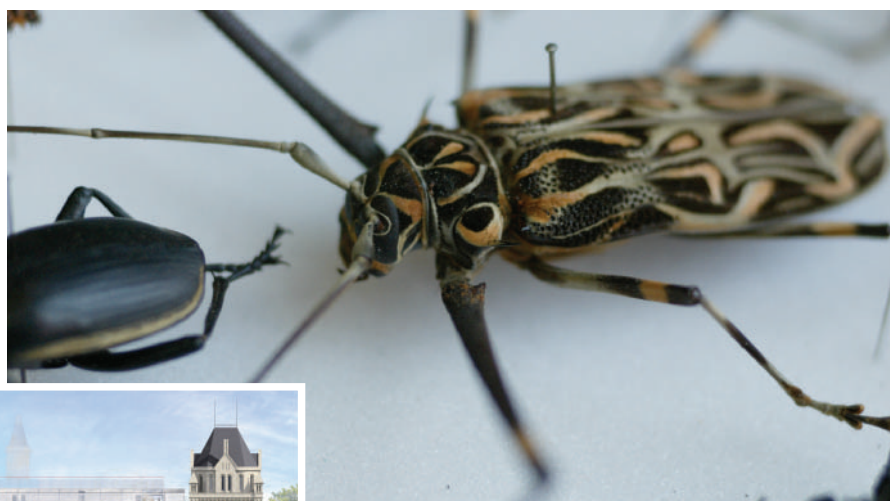
By 2008, the mahogany cabinets that house the 28 million insect specimens at the Natural History Museum in London will be replaced by metal shelving and a sophisticated humidity control system in a striking new building at the museum's South Kensington site. Yet entomologists working on the collection say that the £65-million (US\$125-million) building won't add much to the quality of science that they do.

According to a confidential two-page memo, a copy of which was given to *Nature*, the new facility will be too

small and poorly equipped to deal with advances in information technology that are expected to transform entomology over the next decade. A lack of thorough consultation with scientific staff during the design process has meant that their need for space to work on the collection has been ignored for "reasons of aesthetics", says the memo, which was written by Quentin Wheeler, head of entomology at the museum, and sent on 1 December.

The dispute, in which many museum staff have complained publicly and privately about the relocation plan, is a setback for the museum's extensive expansion programme. The first phase of the plan — a £30-million home for the zoology collection known as the Darwin Centre — opened in 2002, and attracted widespread praise for the way it opened up the museum's stores and research labs to public view. Senior staff say that Darwin Centre II (DCII) will do the same for the entomology collection, as well as providing a better environment for long-term storage.

At DCII, a cocoon-like structure, nestling inside an external glass frame, will house the specimens and provide views of the labs and stores. The samples will be preserved by excluding daylight, and pest control will be enhanced by keeping the temperature low. The current building is saturated with



Pinned down: the planned Darwin Centre II will be home to millions of insect specimens such as this harlequin beetle.



naphthalene, which provides good pest control but is also highly flammable.

Despite these advantages, some entomology researchers and curators describe the move as a "retrograde step" for research. They claim that there is not enough space set aside for several parts of the collection to be examined simultaneously in taxonomy projects, for example. Wheeler's memo also suggests that DCII cannot be adapted to provide space for new "cyber-infrastructure", such as facilities for specimens to be put under a microscope for viewing via the Internet.

Under wraps

In addition, the museum has been unable to confirm that all of the collection will stay in South Kensington. Researchers fear that some specimens will be stuck at a warehouse in Wandsworth, south London, which will be the collection's home while the current storeroom is demolished to make way for DCII. Some entomologists at the museum also allege that the museum is trying to suppress debate and stop them talking to the press. "The museum wants to say everything is rosy," says one member of the scientific staff, who declined to be identified. "It isn't."

Wheeler says that he has had several meetings with senior museum staff since writing

his memo and is confident that the issues he raised can be addressed before the collection move begins in April. He adds that he now understands the design better and that he retracts his comment that it favours form over function. Acknowledging that access to collections may be less easy in DCII, he says he has come round to the idea that the planned design is justified. "We need the best storage. That's at odds with the scientists' need for access," he says. "But you can't optimize both."

Such arguments might persuade entomologists that DCII is the right place for their collections, but researchers and curators are still anxious about the interim storage plans. The warehouse in Wandsworth has higher humidity levels than the museum, raising the chance that common pests such as booklice will attack the specimens. "We're working to bring the humidity down," says Wheeler. "I'm confident it will be addressed."

Richard Lane, the museum's director of science, defends the DCII plans, saying that the critics are just accustomed to their way of working. "In many cases the argument is a surrogate for concern about change," he says. "People now live among the collections. In the new building, the offices will be separate and people are apprehensive about the change in their working environment." ■

Californians hash out guide for spending stem-cell billions

Jonathan Knight, Irvine

The authors of California's plan to spend \$3 billion on human embryonic stem-cell research met this week to work out how the money should be distributed. The group has always said it plans to create a miniature version of the National Institutes of Health; the meeting gave it a taste of how hard that is likely to be.

On 6 and 7 December, experts on grant-making, facilities management, research ethics and intellectual property met at a conference hosted by the National Academy of Sciences in Irvine, California. They produced a lengthy list of details to be hammered out before any grants can be awarded.

The grant money will come from Proposition 71, a measure that was designed to fill a funding gap left by federal limitations on stem-cell research. The initiative, which passed on 2 November, establishes the California Institute for Regenerative Medicine, but leaves details of how the institute will be run to committees that have yet to be formed. State officials must nominate members of the supervisory committee by 13 December — 9 out of 29 had been chosen when *Nature* went to press — and three advisory committees must be appointed by mid-January.

Some decisions will need to be made quickly. Denis Baylor, senior scientific officer at the Howard Hughes Medical Institute, wanted to know whether grants would be awarded on the basis of the merits of the researchers, as his institute's are, or on the details of proposals. "Do you fund people, or projects?" he asked.

Several of the 150 audience members voiced concerns about decisions that have already been made. Nicole Dicks, finance chief of California's Burnham Institute in La Jolla, says she is bothered by fine print that might make life hard for small institutes like hers. Normally, at least one-third of a grant goes to funding facilities and administration costs, but Proposition 71 caps overheads at 25%. Unless this changes, only organizations big enough to pay the difference will be able to accept Proposition 71 money, she says.

Robert Klein, the Palo Alto real-estate developer who was the prime mover behind the initiative and is a likely candidate to chair the supervisory committee, remains undaunted. "These aren't pitfalls, they are challenges," he told delegates. ■



Hand over: some disgruntled researchers are glad to see US health secretary Tommy Thompson depart.

Thompson cedes crown after stormy reign over US health

Erika Check, Washington

With the resignation of Tommy Thompson, who has served as US health secretary since 2001, life scientists in the United States say they hope their sometimes fraught relationship with the government will improve.

In a stormy four-year tenure, Thompson often clashed with researchers at the National Institutes of Health (NIH), the main US biomedical research agency. Although part of Thompson's Department of Health and Human Services, the NIH has traditionally enjoyed considerable autonomy from it.

On 3 December, Thompson said he would step down from his position in February, and hopes to find work in the private sector. His successor is widely expected to be Mark McClellan, a physician and former commissioner of the Food and Drug Administration, who currently runs the health department's largest public-health programmes — Medicare and Medicaid.

Under Thompson, a former governor of Wisconsin, researchers at agencies such as the NIH and the Centers for Disease Control and Prevention (CDC) often chafed at what they saw as his aggressive, political approach.

Soon after he took office, for instance, Thompson irritated scientists at the NIH and CDC by proposing restrictions on travelling to scientific meetings (see *Nature* 411, 983; 2001). Thompson also sought to centralize certain functions of the health department, including press and congressional relations — a move that some researchers said risked politicizing the public outreach of the research agencies (see *Nature* 415, 250; 2002).

His office further drew researchers' ire by limiting the number of health-department employees who could attend the XV International AIDS Conference, held this July

in Bangkok (see *Nature* 430, 128; 2004).

Thompson's supporters counter that his high profile helped the department take the lead on national and international issues: funding for bioterrorism research and global AIDS programmes increased dramatically during his tenure.

Judith Auerbach of the American Foundation for AIDS Research remains critical of Thompson. "We hope the next secretary can avoid instances of politics and ideology influencing public-health research policy, as has happened during this past administration," she says.

"I don't think you're going to find very many people at the NIH who are doing anything but jumping for joy" at his departure, said one senior NIH scientist and administrator, who spoke on condition of anonymity.

As Thompson departed, he defended some of the Bush administration's policies, such as the restriction of federal funds for embryonic stem-cell research. But he also offered blunt warnings about a potential flu pandemic, and the risks of a bioterrorist attack on US food supplies. He praised Elias Zerhouni, director of the NIH, for his handling of a conflict-of-interest scandal at the agency.

NIH researchers were upbeat about McClellan's possible nomination, pointing to his medical degree and strong administrative track record. "I'm hopeful that McClellan understands the importance of biomedical research; he seems to be someone who likes to get things done," said an NIH administrator, who also asked not to be named. ■

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Ecologists attack plans for rare-species act

Emma Marris, Washington

Buoyed by their election victory last month, Republican leaders are gearing up for a push to reform the Endangered Species Act in 2005.

But environmental groups — and many ecologists — fear that the changes could gut the landmark 1973 law, which protects species' habitats throughout the United States.

At a meeting in San Diego on 2–3 December, governors of the western US states discussed the future of the act. These states have the wildest landscapes and the largest number of endangered species in the country. They also have the strongest political tradition of landowners battling with the government over land-use rules.

The meeting was attended by Richard Pombo (Republican, California), chair of the House resources committee and a key figure in the reform effort. "There is a groundswell that next year will be the year to get something meaningful accomplished," says Pombo's spokesman, Brian Kennedy.

The Endangered Species Act is administered by the Fish and Wildlife Service (FWS). It maintains a list of more than 1,000 threatened and endangered flora and fauna, protects the 'critical habitat' in which they live and, when it can afford to do so, leads recovery efforts such as breeding programmes.

Pombo and Senator Mike Crapo (Republican, Idaho), chairman of a Senate subcommittee on fisheries, wildlife and water, have jurisdiction over the act, and both are planning legislation to amend it next year.

Pombo intends to revive two bills that were passed by his committee this year but



Efforts to get the sage grouse listed as endangered are in trouble.

never reached the full House for consideration. One would reduce the amount of land designated as critical habitat, and the other would make the scientific process for listing a species more explicit.

Critics say that both moves aim to disarm the act. "It's not as if people go around wantonly protecting species that don't need protection," says Jamie Clark, who ran the FWS from 1997 to 2001 and is now vice-president of the Washington-based group Defenders of Wildlife. Clark says that the reforms are based on the idea that the science of species conservation is "black and white — which it just isn't".

Crapo's bill is expected to loosen existing deadlines on the designation of species as threatened or endangered and to place restrictions on the designation of critical habitats to protect some species.

Supporters say that the bills will realign

the act so that officials can spend more time saving species. "We want the FWS spending less time in courtrooms and more time out in the field with muddy boots," says Kennedy.

But environmental groups are uneasy. "I have grave concerns that the Endangered Species Act is in peril," says Susan Holmes, a lawyer for the pressure group Earthjustice, based in Oakland, California.

Last weekend's meeting coincided with an announcement by the FWS that it has been advised not to list the greater sage grouse (*Centrocercus urophasianus*) as endangered. Critics regard the FWS's handling of the sage grouse case as evidence that it is softening its enforcement of the species act.

The prospects of the planned reform will depend on how sweeping it attempts to be, political analysts say. Modest reforms will win easy assent, but no one is sure how the new Congress will respond to more extensive changes, such as those backed by some of the western governors. "It's too soon to tell what will happen," says Clark. "People are still working through the politics of it."

The only thing that all sides agree on is that the FWS hasn't got enough money to administer the act properly. The shortfall makes it impossible for the agency to stay on the right side of the existing law, resulting in perpetual legal wrangling over the act. Patrick Parenteau of Vermont Law School in South Royalton, a special counsel for the FWS, says that morale there is at an all-time low. Pombo and his allies promise to work for more funding for the service — if their reforms pass first. ■

Europe faces call to ban imports of wild birds

Rex Dalton, San Diego

More than 200 environmental groups are asking the European Union (EU) to introduce a permanent ban on the importation of wild birds.

The groups plan to file a 12-page declaration with the European Commission by 10 December, requesting the ban in the interests of human health, animal welfare and wildlife conservation.

"When you put the three issues together," says Jamie Gilardi, director of the World Parrot Trust and an organizer of the declaration, the importation of wild birds "makes absolutely no sense".

The trust and some of the other groups

have sought a ban on the wild-bird trade for years, but they are hoping that current fears of a bird flu pandemic in humans will lead the commission to implement one.

The EU temporarily banned importation of all birds — including processed poultry and wild species — from nine Asian counties last January after outbreaks of avian flu in southeast Asia. That ban is set to expire on 15 December, but EU officials say it is likely to be extended until 31 March 2005.

As well as being home to the European Commission, Brussels is the centre of Europe's bird trade. Alberto Laddomada, an EU veterinarian who administers animal-

health issues there, says that the policy of putting wild birds into quarantine twice — once in the shipping nation and once in the receiving nation — is effective at halting the spread of disease. "We can't reduce the risk to zero," he says "but our rules are rigorous and provide a high level of protection."

But campaigners say that Europe's arrangements for enforcing its existing ban are inadequate. And Gilardi says that his trust has uncovered instances in which bird dealers get round the existing system by, for example, falsely claiming that their birds have been quarantined in other countries. "The quarantine facilities in Brussels are a joke," claims Gilardi. ■

Inquiry hears claim of threats over lab move

Jim Giles, London

Some of Britain's most eminent academics are embroiled in an unseemly public squabble over plans to relocate a prestigious London research institute.

At a House of Commons select committee inquiry on 1 December, neuroscientist Colin Blakemore, head of the Medical Research Council (MRC), was accused of inappropriately influencing the task force investigating site options for the National Institute for Medical Research (NIMR). One task-force member said that Blakemore threatened his job if he opposed plans to relocate the institute. Blakemore denies the allegations.

The future of the NIMR has been hotly debated for the past two years. Some, including Blakemore, argue that the facility should move from its current home in Mill Hill, a suburb of London, to a site closer to a London university and hospital, to speed the translation of basic science into clinical advances. But most NIMR staff say they could improve external collaborations without moving.

A ten-member task force was recruited in June 2003 to study the options. This July, they issued a report backing a move to a London university provided the partnership would be better than if the facility remained in Mill Hill. But NIMR staff say that a briefing note



Researchers at Mill Hill oppose being uprooted from their current labs.

on this report was sent to the MRC's ruling council in July stating that the institute's future lay either with King's College London or University College London, ignoring the option of staying put. Blakemore counters that NIMR staff feelings were explained to the council in another document.

The House of Commons Select Committee on Science and Technology launched an inquiry in October into the matter after hearing complaints about this and other events.

One NIMR researcher on the task force, developmental geneticist Robin Lovell-Badge, testified that Blakemore called him and said, "I don't know how you can disagree with me, I am your employer", which Lovell-Badge

interpreted as a threat. Blakemore "absolutely denies" making any such statement.

Blakemore feels that the allegations are part of a campaign to discredit him and hence affect the decision of the council, which has so far backed a move. "I'm shocked that they feel my career is expendable if it means they get to stay at Mill Hill," he says.

Blakemore presented the inquiry with a document, signed by most members of the task force, stating that its work was "properly conducted" and that the panel had been "united". One member, Paul Nurse, who is president of Rockefeller University in

New York, says the phrase "without coercion" was removed from an earlier version of this document in an attempt to convince more members to sign it — although Lovell-Badge and another NIMR worker still did not sign. Nurse says the task-force discussions were closer to "healthy persuasion" than "coercion". Other task-force members agree. "Blakemore behaved with integrity throughout," says Steve Tomlinson, deputy vice-chancellor of Cardiff University.

The select committee is due to meet again on 8 December to decide whether to continue the inquiry. The MRC will consider sites for the institute on 15 December, although a decision is not expected until next year. ■

Move afoot to lend bioweapons treaty more muscle

Declan Butler, Paris

An international rapid-reaction unit to investigate bioweapons incidents is being discussed this week at a meeting in Geneva of the parties to the Biological Weapons Convention (BWC).

The treaty, which has been in force since 1975, outlaws states from developing, stockpiling or using biological weapons. But efforts to give it a mechanism for checking whether states comply with its terms were blocked by the United States in 2001 (see *Nature* 414, 675; 2001) and are still in limbo.

With no broad verification scheme expected any time soon, supporters of tougher checks are trying to promote less ambitious alternatives. The proposed rapid-reaction unit, for example, which is being championed by Britain, would be limited to fact-finding missions after an alleged bioweapons incident had taken place. It would have no powers to investigate allegations that a state

or plant was manufacturing bioweapons.

A rapid-response unit would at least give the BWC some teeth, its advocates say, and planning for it will force the treaty's 152 parties to discuss technical and other issues relevant to wider verification procedures. This would include drafting lists of experts, equipment and transport, as well as agreements on procedures for handling samples and carrying out laboratory tests.

Angela Woodward, a disarmament specialist at the non-profit Verification Research, Training and Information Centre based in London, points out that Kofi Annan, the director-general of the United Nations (UN), already has powers to call for investigations of alleged chemical or bioweapons uses under a 1989 UN law.

The law was developed with chemical weapons in mind, and has become largely redundant since the Chemical Weapons Convention was equipped with its own verification procedures in 1997. But it

could be resuscitated relatively simply, Woodward says, and adapted for bioweapons fact-finding missions.

A working paper drafted by UK experts agrees that the law has not "been reviewed or updated" since 1989. "It is therefore now time to re-establish an effective United Nations procedure for investigating allegations of biological weapons use or suspicious outbreaks of disease," the paper says. It argues that the BWC could guide an update of the law to take account of the specific needs of a rapid-response unit.

This week's meeting is one of three annual summits intended to keep discussion on bioweapons alive before the next major negotiations on the BWC in 2006. It will discuss the rapid-response proposal but will not take a decision on whether to implement it. Advocates hope, however, that the meeting will pave the way for putting the proposal on the agenda at the UN general assembly next year. ■



Peak practice: geologists will carry out a ground-breaking project in a remote part of the Himalayas.

India and China mount joint quake study in the Himalayas

K. S. Jayaraman, New Delhi

The region of the Himalayas where Indian and Chinese troops fought 42 years ago is set to be the site of another meeting between the two nations — this time of their geologists.

The geologists will carry out a two-year study of a part of the mountain range called the Eastern Himalayan Syntaxis. This region, comprising southwest China, northeast India and northern Myanmar (see map, right), is so physically and politically inaccessible that it remains poorly studied by ground-based research.

This is about to change, thanks to an agreement between India's Department of Science and Technology and the Chinese Academy of Sciences, forged by meetings in April and August of this year.

"This region is vital to both India and China as most of the severe earthquakes in this part of the world come from here," says Baldev Arora, director of the Wadia Institute of Himalayan Geology in Dehra Dun, India. He will coordinate the study with Bai Denghai of the Institute of Geology and Geophysics in Beijing, China.

The area is prone to earthquakes because of interactions between the tectonic plates holding India and China, which collided to force up the bulk of the Himalayas, and a block of land including southeastern China and Myanmar. The main collision has been well studied, says Bai, but little is known about the interaction of the plates with the land to the east.

Some studies suggest that the Himalayas are overriding southeastern China. "This is one thing we want to confirm," says Bai. The geologists will also try to verify whether a 'plume' of heat from deep within the mantle wells up beneath the region, as some Chinese scientists have suggested.



Most of the existing information about the Eastern Himalayan Syntaxis comes from satellites, or from seismometers placed hundreds of kilometres away. The joint study will supplement this with geophysical and geological field data to help construct a three-dimensional model of the region.

Instruments will be placed in a corridor running from Arunachal Pradesh in India, through Myanmar to southwestern China. Scientists will collect data in their own section of this corridor, except in Myanmar, where agreements will allow Indian and Chinese scientists to work side by side. "Once the field measurements are done, the data will be processed jointly to obtain a unified model of the collision zone," says Arora.

This arrangement may sound less than ideal for a joint project but, given the political circumstances, it is a "practical approach," says Arora. He thinks the level of cooperation will increase once the proposed Joint Steering Committee, co-chaired by science ministers of the two countries, is put in place early in 2005 (see *Nature* 432, 427; 2004).

Details of the study are set to be finalized at a meeting in February, with the work expected to be completed in 2007.

Grads rally round student in Toronto harassment case

Erika Check, Washington

Canadian graduate students have voiced support for a complaint against the University of Toronto, Ontario, about its handling of a sexual harassment case.

On 27 November, Canada's National Graduate Caucus passed a motion of support for claims filed in May by Gwen Schwartz with the Ontario Human Rights Commission (OHRC). Schwartz alleges that her former graduate adviser sexually harassed her and that she suffered reprisals when she raised the issue with administrators. She also alleges that she has been blocked from retrieving her data from her former adviser (see *Nature* 430, 711 and 958; 2004). The OHRC is considering a formal investigation of her complaints about the university and its affiliated hospital group, the University Health Network (UHN).

The caucus, a component of the Canadian Federation of Students, represents about 60,000 graduate students. Its motion supports Schwartz's OHRC complaints and calls on the university to help her regain her data.

"It's important that Gwen knows that she has support," says Rose Da Costa, resource coordinator for the University of Toronto Graduate Students' Union. "She has gone through a very serious situation and has not achieved a satisfactory resolution." Da Costa raised the motion of support at the national caucus after successfully passing a similar motion at Toronto.

Both the hospital and the university believe that a settlement they negotiated with Schwartz and several other parties last year, after a mediation process, resolved the issues. Schwartz disputes the settlement, claiming that she does not agree with its terms and felt pressured to sign it. However, both the UHN and the University of Toronto consider it final and binding. Because of the possible OHRC investigation, UHN lawyers said the hospital would not comment for this report.

The University of Toronto also said it would not comment on the caucus's motion. However, it "vigorously denies" Schwartz's allegations and says it has always acted appropriately. "The university has met all its obligations under the agreement, including financial and academic support for Ms Schwartz to enable her to move forward with her studies," says Angela Hildyard, vice-president of human resources.

US panel stirs debate about feasibility of cold fusion

Washington Claims for cold fusion are intriguing but unconvincing, says a review panel of the US Department of Energy.

Fifteen years after Stanley Pons and Martin Fleischmann of the University of Utah announced that they had created nuclear fusion in a test tube at room temperature, cold-fusion advocates were given a chance to have their work reviewed.

David Nagel, from George Washington University in Washington DC, who co-authored the summary of research that

the panel reviewed, says: "Most scientists think that cold fusion is laughable, but when the dust settled, the researchers reviewing our work were evenly split."

The 18-member panel was divided "approximately evenly" on the question of whether any experiments have produced power in the form of heat. But members agreed there is not enough evidence to prove that cold fusion can occur, and complained that much of the published work was poorly documented.

UK to relax tax stranglehold on enterprising academics

London Britain will fix the tax laws that may be stifling the creation of spin-off companies by universities, chancellor Gordon Brown announced on 2 December.

The current rules mean that scientists who create companies are obliged to pay hefty taxes on whatever the Inland Revenue judges to be the value of their shares, even if they have yet to make a profit. Formulated to close a tax loophole, the rules have slowed down the creation of companies by UK universities (see *Nature* 429, 6; 2004).

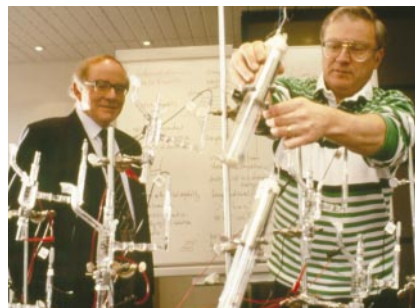
Speaking at the launch of his pre-budget report, Brown said the rules would be scrapped and universities consulted on an alternative legal framework.

The chancellor flagged two further science initiatives. Manchester, Newcastle and York have been designated 'science cities' and will share £100 million (US\$190 million) to boost university-business collaborations. And the treasury will pilot a scheme aimed at increasing university independence, in which the government will match the funds that institutions raise for their endowments.

Europeans rewarded with Descartes prizes

Munich The winners of the European Union's newly created Descartes Prize for Science Communication were announced on 2 December at a ceremony at Prague Castle in the Czech Republic.

Britain's David Attenborough was one of five winners to share the €250,000 (US\$340,000) prize for his long career as a wildlife film-maker, and French film producer Vincent Lamy won a share for a documentary on stick insects. Nanoscientist Wolfgang Heckl, director-general of the Deutsches Museum in Munich, won for his commitment to explaining science to the public. Peter Csermely, head of the Molecular Chaperone Lab of Semmelweis University, in Budapest, was rewarded for creating the Hungarian Research Student Movement, which gives students from underprivileged



Cool reception: Stanley Pons (right) and Martin Fleischmann's cold fusion remains unproven.

families access to top labs across Hungary. And Ignaas Verpoest, a Belgian materials scientist, won a share of the prize for a travelling roadshow on composites.

The €1-million Descartes Prize for Research was awarded to collaborations working on quantum cryptography and mitochondrial diseases.

Female 'passion patch' turns off FDA safety panel

Washington A testosterone patch for women was unanimously rejected last week by an advisory panel of the US Food and Drug Administration (FDA). The Proctor & Gamble product, Intrinsa, was expected to be approved for use by women who have lost their libido after the removal of their ovaries.

Panel members said they were concerned that the hormone might increase the risks of cancer and heart attacks, and called for larger and longer clinical trials to verify the patch's safety. Industry watchers speculated that the caution stemmed less from the safety and efficacy of the data in hand than from nervousness at the FDA following recent safety controversies, even though the advisory panel is independent of the agency (see *Nature* 432, 537; 2004). The FDA is not obliged to take recommendations from its advisory panel, but usually does.

Angola trumpets mine-clearing mission

Paris A project was launched last week to clear mines sown during three decades of Angolan civil war and open an old elephant migration route linking Botswana with Zambia and Angola.

Botswana's growing elephant population is threatening agriculture and vegetation, and reopening the route is the only way to avoid massive culling. The US\$1-million project is funded by Roots of Peace, a California-based group that works to turn minefields into farmland. The plan was announced last week at the Nairobi Summit on a Mine-Free World.

"The threat to people from these seeds of misery must be our first concern but it is clear



that the environment, upon which local people depend for food, shelter and natural medicines, suffers too," says Klaus Toepfer, executive director of the UN Environment Programme.

Row over axed courses hits UK government

London The sound of axes falling on British science departments has finally reached the government's ears. Charles Clarke, the education secretary, wrote to the Higher Education Funding Council for England last week, asking what "encouragement and incentives" could be made to protect subjects of "national strategic importance" that are at risk, including chemistry, physics and maths.

David Giachardi, chief executive of the

Royal Society of Chemistry, called the statement "a bit thin and a bit late". For example, the University of Exeter recently decided to close its chemistry department (see *Nature* 432, 543; 2004) and the University of Newcastle upon Tyne is to abolish its pure physics degrees.

Correction

In our News Feature "David versus Goliath" (*Nature* 432, 546–548; 2004), we mistakenly switched Watson and Crick's training: Watson was the zoologist, and Crick started as a physicist.

Studies of faith

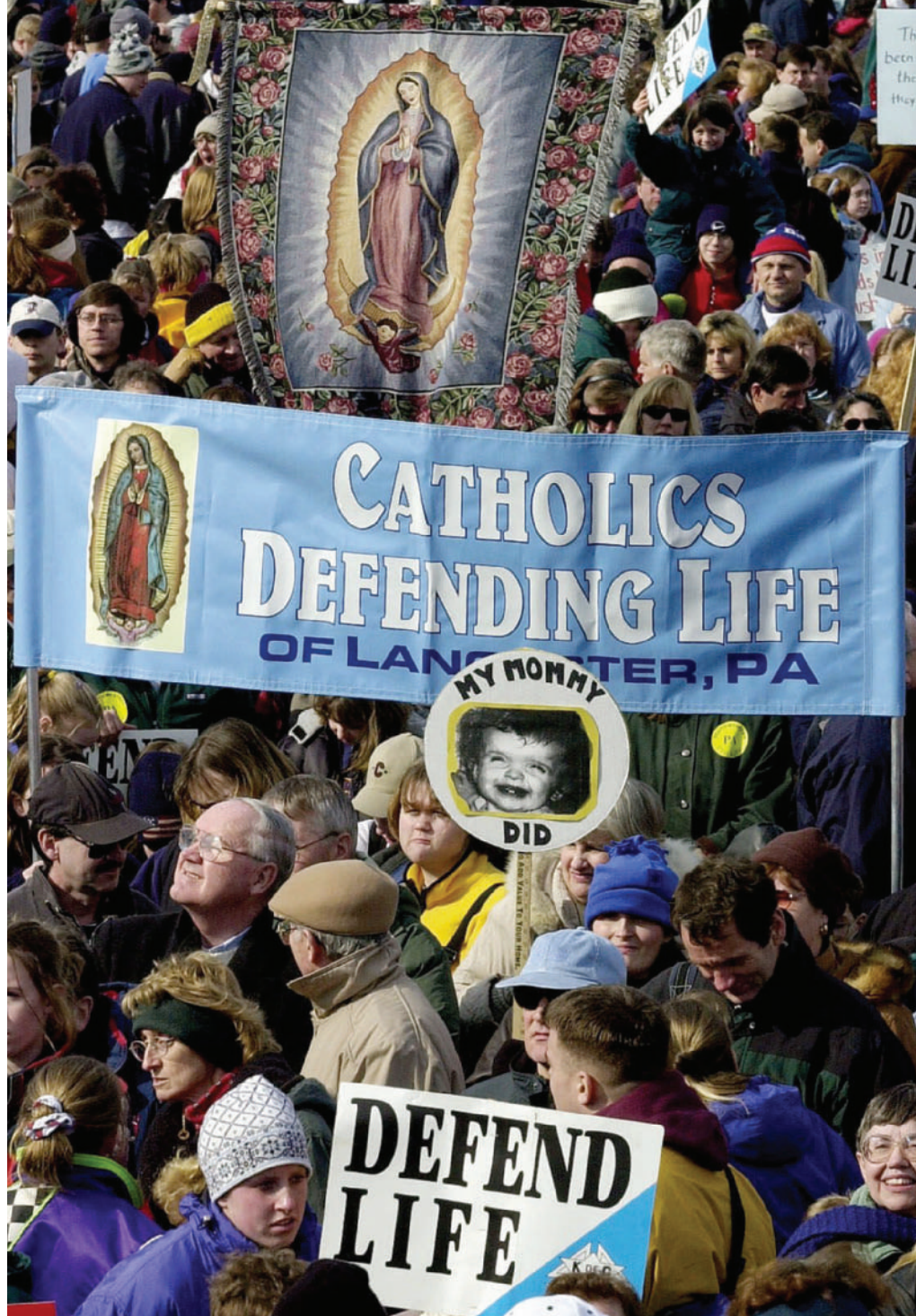
Embryonic stem-cell research is putting fresh strain on the already fractious relationship between science and religion. Tony Reichhardt explores how faith is shaping the ever-changing landscape of bioethics.

When Pope John Paul II addressed the Pontifical Academy of Sciences in 1992, he tackled yet again Galileo's famous battles with the Church four centuries ago. In his talk, entitled "Faith can never conflict with reason", the Pope was doing his best to mend fences. Although science and religion form "two realms of knowledge", he said, "the two realms are not altogether foreign to each other, they have points of contact".

Despite the Pope's optimistic words, the tension between faith and science never fully subsides. And as these realms regularly come into contact, over everything from Darwin to Dolly the cloned sheep, they sometimes collide with explosive force.

Today, with scientists manipulating the machinery of life as never before, the debate is in full swing. Nanotechnology, artificial intelligence, cloning, creationism and genetic modification (see 'A recipe for disaster?', opposite) all test the strained relationship between faith and advancing technology.

Today's frontline controversy — stem-cell research — has prompted a wide range of reactions from religious leaders, much of it negative. But the fundamental, religion-based belief in the sanctity of human life, even at the stage of an embryo, clashes in this field with another fundamental human desire: to alleviate suffering and cure disease. The debate does not leave room for simple answers, for individuals or society as a whole. Francis Collins, head of the US National Human Genome Research Institute in Bethesda, Maryland, and a devout Christian, has described himself as being "intensely



Ethical balance: Christianity's defence of the unborn child informs its position on stem-cell research.

conflicted" over stem-cell research. "It is a classic example of a collision between two very important principles," he says. The opposition to stem-cell research cannot be dismissed as merely 'anti-science'. Most religious traditions sincerely value medicine and science, and make a serious effort to reconcile scientific thinking with doctrine (see 'Science and the Vatican', page 669).

Where there's life...

This process of discussion and reconciliation may even be initiating a fundamental change. Some Catholics are beginning to hope that recent insights into developmental biology could move the Church from its 135-year-old position that human life

begins at conception — the main obstacle to it accepting the study of stem cells extracted from human embryos.

Much of the theological debate about stem-cell research centres on the question of when life begins. Some traditions, including most sects of Judaism and Islam, aren't troubled by this because they don't consider the early embryo fully human. Most Jewish Talmudic scholars, for example, argue that 'ensoulment' takes place 40 days or more into pregnancy, once the human form is roughly established. Before that, the embryo is described as 'water'. Israel accepts embryonic stem-cell research, and the Israeli Academy of Sciences and the Jewish Rabbinical Assembly, headquartered in the United

States, have both come out in favour. Likewise, researchers at the Royan Institute in Tehran have developed stem-cell lines with the full blessing of Iran's supreme leader, Ayatollah Ali Khamenei.

According to Hinduism, life begins at conception. But this does not make for easy decisions on the ethics of stem-cell research. Destruction of an embryo could still be justified if it is considered to be an "extraordinary, unavoidable circumstance" and an act "done for greater good", says Swami Tyagananda, Hindu chaplain at the MIT Religious Activities Center in Cambridge, Massachusetts. Based on these criteria, many traditional Hindu priests are unwilling to condone the work, but it has not provoked much opposition in India, for instance, where embryonic stem-cell research is allowed.

The strongest objections come from Christian sects that regard the sacrifice of an embryo — even an undifferentiated clump of cells in a three-day-old blastocyst — as totally unacceptable. Embryos cannot be killed, they say, any more than Death Row prisoners can be used in lethal experiments, even if the goal is to relieve suffering in others.

Evangelical Christianity relies on a specific interpretation of scripture for its advice on this matter. Psalm 139: 13, for example, says: "For you created my inmost being; you knit me together in my mother's womb." In Jeremiah 1: 5 God tells the prophet, "Before I formed you in the womb I knew you," implying that Jeremiah had 'personhood' in God's eyes even before he was an embryo.

This roughly matches current Vatican thinking. The Catholic Church holds that human life is sacred from the moment of fertilization. But some Catholic theologians point out that the Church's view on the moral status of the embryo has changed over time, and may change again. In fact, scientific breakthroughs — the discovery of the

mammalian ovum in 1827 and the first microscopic views of developing embryos — helped to shape the Vatican's thinking. The findings informed Pope Pius IX's decision in 1869 to abandon the Church's moral distinction between early- and late-term abortions and to call instead for full protection of life from the moment of conception.

Today, the Vatican does not strictly claim that the early embryo is a person — only that it deserves respect as a potential human being, says Carlos Bedate of the Autonomous University of Madrid. Bedate has an unusual background as a Jesuit priest with a doctorate in molecular biology, and has served on a Spanish advisory committee for bioethics.



Religious views on stem-cell research hinge on when life begins: from conception to when the embryo looks human.

He thinks the ambiguity in Catholic thought could open a window for the Church's acceptance of embryonic stem-cell research. Recent advances in developmental biology have shown that an embryo's viability depends on the cellular environment as well as its own DNA, says Bedate. "We cannot consider that in the early embryo there is the entire information needed to complete the process of development," he says.

Open debate

A few Catholic theologians have spoken out in favour of human embryonic stem-cell research, including Jean Porter of the University of Notre Dame in Indiana, Margaret Farley of Yale University, and Christian Kummer, who trained as a zoologist and is now

director of an institute for scientific issues related to philosophy and theology at the Jesuit Faculty of Philosophy in Munich. Kummer says that they are free to voice these views without fear of censure from the Church. "Academic freedom is more pronounced than one would expect from knowing the Vatican's official positions," he says.

Bedate thinks that the Vatican may eventually be open to reconsidering the issue on the basis of new scientific understanding. But any formal change in the Church's position is likely to come very slowly, as Galileo's case once showed.

Arguments about the moment of ensoulment are crucial, but they are not the only

factor in religion-based objections to stem-cell research. As evangelical Christian Nigel Cameron, a bioethicist at the Institute on Biotechnology and the Human Future in Chicago, Illinois, told a US Senate committee in 2001: "It is by no means necessary to take the view that the early embryo is a full human person in order to be convinced that deleterious experimentation is improper."

The Church of England, for example, does not contend that early embryos are fully human. Yet they are "deserving of respect" nonetheless. In its guidelines on ethical investment, the Church concludes that "companies, a major part of whose business is engaged in the cloning of embryos (even for therapeutic use), should be avoided."

Another point of controversy is the source

A recipe for disaster?

If ever there was a technology that could get mankind accused of playing God, it is genetic engineering. As humanity mixes species with species and creates clones of creatures, religions have had to grapple with questions that could not have been foreseen when most religious texts were written.

The Bible can be used to argue both for and against genetic manipulation. In the Old Testament, lines such as "Thou shalt not sow thy field with mingled seed" (Leviticus 19: 19), and "Thou shalt not sow thy vineyard with divers seeds" (Deuteronomy 22: 9), have been used by some Christians as evidence that meddling with creation is unacceptable — although the same lines could be used to argue against traditional agricultural

practices. Others note that, according to Genesis, man was made in the image and likeness of God and given dominion over all living things, which would make genetic modification mankind's right.

The Catholic, Jewish and Islamic faiths generally see no need to ban genetically modified foods or vaccines for moral reasons. On a purely practical level, Jews and Muslims share the worry of whether their food contains any hidden genes from pigs or other forbidden dietary products.

In the Hindu tradition, food is believed to affect your physical and mental constitution and your karmic balance, says Cromwell Crawford, an expert in Hindu bioethics at the University of Hawaii at Manoa. Each Hindu must assess whether the motivation behind genetic engineering is a positive

desire to help feed the world or a negative one driven by commercial exploitation, he says, in order to determine its impact on body and mind.

Other religions also share this worry about how genetic engineering is used. But on the purest level of assessing the ethics of the technology itself, there is little to go on to decide whether mixing corn with bacteria is ok, but mixing mankind with mice is not. "There's no list of boxes to tick off to tell you when you have gone too far and changed the animal or plant into something fundamentally different," says Donald Bruce, director of the Church of Scotland's Society, Religion and Technology Project. "There is no commandment from God saying: 'Thou shalt not genetically engineer.' It's a judgement call."

Zeeya Merali



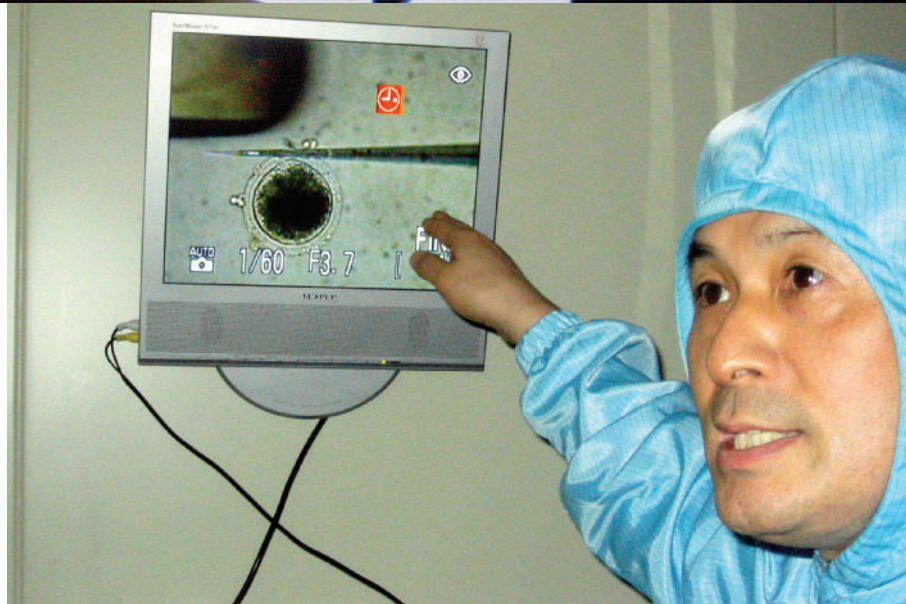
Opposite sides: many US Christians cannot condone research using embryonic stem cells (above), whereas Woo Suk Hwang in Seoul claims that his research is in line with Buddhism.

of the embryo. Some stem-cell researchers use embryos discarded from *in vitro* fertilization (IVF) clinics, whereas others clone new embryos to harvest their cells.

The very practice of IVF has faced strict opposition from the Catholic Church on the grounds that it breaks the God-given connection between sex and procreation — a rule often voiced during discussions on the ethics of contraception. Most other religious groups, including evangelicals, see IVF as a good solution for infertile couples who want children. But that acceptance is now coming under greater scrutiny because IVF clinics frequently discard ‘excess’ embryos that are not needed for implantation. Although the issue hasn’t received the same attention as abortion, some Christian leaders have begun to speak out. “IVF kind of snuck up on evangelicals. We weren’t paying as close attention as we should have,” says Ben Mitchell, an bioethicist and evangelical Christian at Trinity International University in Deerfield, Illinois.

Fertile ground

In predominantly Catholic Italy, attempts to find a compromise on the issue have led only to new problems. The country passed a law this year legalizing IVF despite Vatican opposition. But no embryos can be destroyed — all have to be transferred to the mother’s uterus. This can increase the risk to mothers and even lead to miscarriages in the case of multiple pregnancies.



Other denominations, including the Unitarian Universalists, one of the most liberal of religious groups, take more umbrage with using custom-made embryos than the ‘left-overs’ from IVF. Although the Unitarian Universalist Association has no official consensus opinion on stem cells, its president, William Sinkford, offered his personal opinion in 2001 that there should be no ban on embryonic stem-cell research. But he added: “I would contend that no human embryos should be created specifically for stem-cell experimentation, thus turning human life and human reproduction into a commodity — surely a clear affront to our first principle affirming the inherent dignity of human beings.”

Perhaps this dignity seems all the more

affronted since the resulting experiments have not, so far, yielded life-saving results. Damien Keown, a specialist in Buddhist ethics at Goldsmiths College in London, sums it up: “Scientific curiosity seems to be the main factor motivating cloning experiments at present, and overall Buddhists are likely to be sceptical about the need for this curiosity to be satisfied at the price of destroying human life.”

When Seoul National University’s Woo Suk Hwang cloned human embryonic stem-cell lines earlier this year he cited his own Buddhist beliefs, saying that the experiments were a kind of “recycling of life” in line with reincarnation. Some Buddhist groups in South Korea, where Buddhists account for about a third of the population, supported

Science and the Vatican

Marialuisa Lavitrano had been uncertain about what to expect. A pathologist at the University of Milan-Bicocca, Lavitrano had been invited to organize a series of scientific meetings at the Vatican about xenotransplantation and the genetic modification of animals. That was three years ago, and she is still surprised by the response she got.

"The cardinals wanted to know everything about the science," she says. "It was a fascinating debate and, frankly, I was not prepared for so much open-mindedness." After the meetings, the Vatican legitimized the transplant of animal organs into humans and the use of animals in medical research (E. Sgreccia *et al. Nature* **414**, 687; 2001).

Lavitrano's experience is not unique. The Vatican often seeks informed advice on questions emerging from progress in science and medicine. And many researchers who have been involved say that they are surprised by the high quality of scientific discourse with the cardinals. Despite the Church's reputation for nurturing anti-scientific tendencies — as recently as the 1960s, Catholic priests in training were asked to renounce 'modern errors' such as darwinism and the expansion of the Universe — the Vatican has long abandoned literal interpretations of scripture.

The Vatican takes regular scientific advice from the 400-year-old Pontifical Academy of Sciences in the Vatican City. The academy is made up of 80 eminent scientists from around the globe chosen by the academy itself. Each November they hold a scientific meeting, usually on a topic of their

choosing, or sometimes on a matter requested by the central administration of the Church; these have covered everything from birth control to cloning, genetic engineering and the origin of life.

The annual meetings conclude with an audience with the Pope. He and his officials then take this scientific advice into account when they draw up guidance notes and issue decrees on the Church's doctrine.

Pope John Paul II has shown a notable interest in science ever since he took over as head of the Catholic Church in 1978. "Scientists who have met him have always thought they were before a person really interested in their work and sincerely eager to learn from them," says Giuseppe Tanzella-Nitti, an astrophysicist at the Pontifical University of the Holy Cross in Rome.

"I remember the young Pope just a few years after his election sitting among a small group of scientists on a terrace of his private residence at Castelgandolfo, taking notes about contemporary cosmology," says Tanzella-Nitti. Perched on top of the same residence is an astronomical observatory that, partnered with a telescope in Arizona, is funded by the Vatican to the tune of US\$1 million a year.

The possibility of extraterrestrial life and intelligence, and the implications of cosmology for Christian ideas about the beginning and end of time, will be upcoming challenges for science-minded theologians, says Tanzella-Nitti. The Catholic Church, for one, should be well prepared.

Quirin Schiermeier

him. But most Buddhist scholars say the killing of an embryo at any stage violates a central tenet that living things should not be harmed. Cloning for reproductive purposes, on the other hand, does not require destroying the embryo and so does not in itself violate Buddhist precepts.

"The problem is not when life is started, but when it is stopped, as in therapeutic cloning," says Keown. "Dr Hwang is on shaky ground in claiming that Buddhism supports cloning, without careful qualification."

Protestants, who make up another third of South Korea's population, reacted strongly to the news of Hwang's experiments. Sixteen Protestant groups, representing 6,000 people — half of them doctors in the Christian Medical Fellowship — met in September to plan a campaign devoted to making the use of human embryos in research illegal.

But the extent to which religious opinion influences politics and laws varies dramatically from society to society. In Spain, where 99% of the population is Catholic, a law was recently passed to allow the use of IVF embryos in stem-cell research. But in Italy, which vehemently opposes the use of European Union funding for stem-cell research, the population is "much more reactionary on religious issues than the Church itself",

says Enrico Alleva, acting head of behavioural pathophysiology at the National Institute of Health in Rome. Alleva believes that this zeal, rather than formal Catholic doctrine, is at the core of the recent creationist movement in Italy (see *Nature* **428**, 595; 2004) and of other perceived 'anti-science' tendencies throughout Europe.

Body politic

Another place where religion has proved to be the driving force for politics is the United States. In November's election, President George W. Bush owed his victory in part to votes from his fellow evangelical Christians.

Evangelicals are not uniformly conservative in their political views — some oppose capital punishment, for example. But most evangelical leaders are strongly against embryonic stem-cell research. The Southern Baptist Convention, which represents the second largest US denomination after Catholics, says it relies on a "crass utilitarian ethic which would sacrifice the lives of the few for the benefits of the many".

Such statements have surely influenced the United States' rigid policy on stem-cell research, in which federal funding is limited for use on a few dozen pre-established cell lines, and cannot be used to establish new ones.

Does this reflect public attitudes? Polls reveal mixed opinions — although a lot hinges on the wording of the question. In July 2004, Catholics for Free Choice published a poll of 2,239 Catholics nationwide, and found that 72% supported "allowing scientists to use stem cells obtained from very early human embryos to find cures for serious diseases such as Alzheimer's, diabetes and Parkinson's".

But "polls on embryonic stem-cell research often fail to mention that the research requires destroying human embryos", says Richard Doerflinger, deputy director of the US Conference of Catholic Bishops Secretariat for Pro-Life Activities. In August the Catholic bishops released the results of their own poll. When given a choice between funding both adult and embryonic stem-cell research or only work that didn't require destroying an embryo, Americans preferred the latter by 61% to 23%.

Difficult question

Efforts to establish ethical rules on stem cells that transcend national and spiritual boundaries have proved remarkably unsuccessful. After years of delayed decisions, on 19 November the United Nations came to what was widely called a "compromise" position on cloning technologies — it adopted a non-binding declaration that asks member states to adopt legislation that respects "human dignity". In the end, this statement is likely to be interpreted in as many different ways as some lines from the Bible.

So scientists and theologians will continue to talk — and to disagree. At least one thing has changed in this debate since Galileo's day, for better or for worse: now, science is the orthodox worldview, in the industrialized world at least, and religion stands outside, raising objections. At bioethics conferences, says John Evans, a sociologist of religion at the University of California, San Diego, biologists rarely show any knowledge of theology. But "religious people are expected to have spent huge amounts of time learning all the science", he notes.

One thing is certain. Everyone agrees that fundamental ethical questions underlying stem-cell research, many of which transcend religion, need to be addressed. "The power of these new technologies is so great that we can no longer deal with them in a vacuum. This affects everyone across the board," says Kevin FitzGerald of Georgetown University in Washington DC, a Jesuit priest with PhDs in molecular genetics and bioethics. And stem cells are just the beginning. "The stuff that's coming down the pipe will make this look like child's play," he says. "Organic mixed with inorganic, one species mixed with another. Everything from the molecular level on up will be fluid."

Tony Reichhardt writes for *Nature* from Washington. Additional reporting by David Cyranoski in Tokyo and Quirin Schiermeier in Munich.

Buddhism on the brain

Many religious leaders find themselves at odds with science, but the head of Tibetan Buddhism is a notable exception. Jonathan Knight meets a neurologist whose audience with the Dalai Lama helped to explain why.

One of the first things people discover when they meet His Holiness the Dalai Lama is that the head of Tibetan Buddhism likes a good laugh. “He jokes all the time,” says Fred Gage, a neuroscientist at the Salk Institute for Biological Studies in La Jolla, California, who met the spiritual leader for the first time in October. “He has a great sense of humour.”

This is probably a good thing. The occasion for this meeting — a research conference held at the Dalai Lama’s headquarters in Dharamsala, India — included a presentation of evidence that people in good spirits are better able to control their blood sugar levels. Other talks suggested that meditation can transform emotions and that daily experiences can alter the expression of genes. Gage presented his research into how the brain can remake itself throughout life.

It was the 12th time since 1987 that the Dalai Lama has convened leading psychologists and neurobiologists to hear the latest scientific thinking in fields related to the human mind. These meetings are organized by the Mind & Life Institute in Louisville, Colorado, which was established in the 1980s to promote communication between science and Buddhism. But much of the credit for this open communication goes to the Dalai Lama himself.

Spiritual links

In accordance with Tibetan tradition, the current Dalai Lama, Tenzin Gyatso, was recognized as the 14th reincarnation of the Bodhisattva of Compassion in 1937, when he was only two years old. Gyatso has long had an interest in science. When he accepted the Nobel Peace Prize in 1989, he commented: “Both science and the teachings of the Buddha tell us of the fundamental unity of all things.” He once said that if he had not been a monk, he would have been an engineer.

Enthusiasm for science seems to extend beyond the spiritual leader. Tibetans, surprisingly enough, were the most strongly represented ethnic group working on the Human Genome Project: although they account for only 0.1% of the world’s population, Tibetans made up about 10% of the project’s workforce (see *Nature* **425**, 335; 2003).

For many Buddhist monks, this interest in science is focused on an intense curiosity about the workings of the brain. Monks typi-



Science with a smile: the Dalai Lama has a deep personal interest in research developments.

cally spend hours in meditation each day, a practice they say enhances their powers of concentration. Highly trained monks report being able to focus on a single object for hours without distraction and to recall complex scenes in exquisite detail. A question that deeply interests the Dalai Lama, and indeed some neuroscientists, is whether these phenomena have a biological basis.

Gage studies the ability of the mammalian brain to change and adapt in adulthood. Before the late 1990s, it was thought that adult brains were more-or-less complete. Learning involved the development of new connections — but no new neurons were born, and when these cells died they were gone forever. Now it turns out that new neurons do grow and our brains are much more flexible than was once believed. As a key component of Buddhist belief is that meditation literally transforms the mind, Buddhists are keenly interested in scientific advances that could help explain this observation.

Gage’s talk on 18 October in Dharamsala — seat of the Tibetan government-in-exile

since 1960 — kicked off a five-day private conference on ‘neuroplasticity’. Gage gave a general primer on the complexity of the nervous system, and then launched into a two-hour presentation of his research targeted at a lay audience. Next to him, the Dalai Lama listened intently, making occasional use of two interpreters to translate into Tibetan things he didn’t immediately grasp in English. Also in the audience were the six other presenters and a handful of Buddhist monks.

Lessons learned

Although the group did not come to any Earth-shattering conclusions about cognition, they did reach a higher understanding of each other, which was the main point of the exercise. For the monks, the sessions may help them deal with modern questions not addressed in traditional Buddhist teachings, such as the issue of the morality of stem-cell research (see page 666). Scientists in turn have plenty to learn from the monks — after centuries of inner contemplation, Buddhists claim to know a thing or two about how the mind behaves.

Richard Davidson, a psychologist at the University of Wisconsin, Madison, and the coordinator of the Dharamsala conference, has learned from the monks through study. He found that certain neural processes in the brain are more coordinated in people with extensive training in meditation, an observation that may be linked to the heightened awareness reported by meditating monks (A. Lutz *et al. Proc. Natl Acad. Sci. USA* **101**, 16369–16373; 2004).

Gage says that what particularly impressed him was the Dalai Lama’s empirical approach. “At one point I asked: ‘What if neuroscience comes up with information that directly contradicts Buddhist philosophy?’” says Gage. “The answer was: ‘Then we would have to change the philosophy to match the science.’”

So far that hasn’t been necessary. And if the reported benefits of laughter are correct, there is no need for the Dalai Lama to rein in his sense of humour either. During a discussion of how our childhoods shape who we are, he observed that he liked to play with toy guns as a child and even picked on his brother. “I was the mean one,” he said, thereby stabilizing blood sugar levels throughout the room. ■

Jonathan Knight writes for *Nature* from San Francisco.

J. BOURG/REUTERS

Australians net benefits of sustainable fish farming

Issues of concern need to be recognized, confronted through research and resolved.

Sir—Your News Feature “Fishing for trouble” (*Nature* 431, 502–504; 2004) focuses on the potential environmental risks associated with tuna farming in coastal waters off North and Central America. In South Australia, on the other hand, tuna farming is being undertaken in what we believe is an ecologically sustainable manner.

Since 1991, the farming of southern bluefin tuna in South Australia has grown into the largest aquaculture industry (by value) in Australia. Production is currently at about 10,000 tonnes per annum, which is considerably greater than that being produced in Mexico or planned for the United States.

When the industry was first established in Australia, many potential environmental risks were identified. Subsequently, as the industry has developed, researchers have been able to confront some of these early speculative statements with real data and experience. An important part of this process has been the development of a system for identifying and quantifying

risks, which has been delivered through projects within the Cooperative Research Centre for Sustainable Aquaculture of Finfish (Aquafin CRC), set up in 2002.

The Aquafin CRC aims to develop knowledge and technologies to underpin the sustainable growth of the finfish aquaculture industry in Australia and has funding of A\$72 million (US\$53 million) over seven years. This investment comes from the tuna and salmon industries, the Fisheries R&D Corporation, universities, and state and federal research agencies.

In contrast to the fears expressed in your News Feature about spreading disease, we have assessed the health of captive tuna and in all cases the risks ranged from negligible to low under current practices (see www.sardi.sa.gov.au/sbt). Other studies have investigated the impact of tuna farms on the composition of sediments and benthic infaunal communities below the sea-cages. These assessments provide just one measure of the environmental performance of the industry, so research programmes within

the Aquafin CRC are now aimed at identifying the nature and quantities of farm wastes and understanding regional effects, such as the impact of farming operations on silver gulls and other key species, including sharks and pinnipeds.

Although we acknowledge that your News Feature provides information that will inform the debate about potential risks of tuna farming in North America, we would argue that tuna farming (and indeed any finfish aquaculture) can be practised in a sustainable manner. The real strength in the model adopted by the Aquafin CRC and its partners is that these issues, rather than fuelling public concern and criticism about sustainability, are openly and robustly confronted through research and resolved through the development of appropriate mitigation strategies.

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Politics: scientists are as qualified as anyone else

Sir—M. J. Hsu and G. Agoramoorthy argue, in Correspondence, that scientists and teachers should ignore politics (*Nature* 431, 627; 2004), citing Taiwanese Nobel laureate Lee Yuan-tseh as an example of a scientist’s unsatisfactory involvement in politics.

Lee is a highly respected scientist worldwide. This is especially true in his home country, where he has devoted himself to the welfare of the people. In his constant endeavours for Taiwanese sciences, there is not the slightest evidence that Lee has ever been biased by his political preference. It seems to us that citing a signature campaign against him is in itself a political action.

It is pervasive thinking in Taiwan that scientists should not get involved in politics under any circumstances. The main reason, pointed out by advocates of this ideology, is that expertise in science does not guarantee that scientists are masters in politics.

But who does qualify as a master of politics, in reality? Studying politics as an academic discipline is very different from practising it. Meanwhile, this position deprives scientists of their rights to participate in debating public policies.

Scientists can also make a positive contribution when engaged in the political system. They could contribute to a more environmentally friendly energy policy, for example, or security regulations that do not scare away international scholars.

In this regard, Nobel laureates who endorse the politicians they respect should be encouraged, not condemned.

Chun-Liang Pan*, **Ming-Chang Chiang†**

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If we ignore politics, will politics ignore science?

Sir—The recent News stories and Correspondence on the relationship between politicians and academics (*Nature* 430, 595; 431, 1; 431, 627; and 431, 1036; 2004) missed a central issue: will politics ignore the scientists?

Nobel laureates in the United States and Taiwan have recently felt compelled to become involved in presidential politics. The explanation most frequently offered for this phenomenon is simply “It’s their right as citizens”, but the reasons often go deeper than that. Modern scientific and

academic research has become so costly that little of the most cutting-edge research can be accomplished without government approval or support, providing a strong motivation for politicking. To be ignored would be a disaster.

Furthermore, in less democratic societies (such as Taiwan was in the recent past) control over intellectual freedom is well documented, providing a strong reason for resistance.

Only 13 years ago, I was told by one of my students that a teacher-officer had asked him whether I had “talked about politics” in my lectures. Teacher-officers were military officers, stationed at Taiwan’s universities in those days to work closely with the then-ruling Nationalist Party on a variety of duties, which included keeping an eye on political activists on campus.

The recent democratization process in Taiwan has led to a major change in teacher-officers’ duties. But the old political atmosphere remains much in evidence. It is no wonder that your correspondent M. J. Hsu, who is currently at Taiwan’s National Sun Yat-sen University (*Nature* 431, 627; 2004), should wish that we could all just ignore politics.

Ying-Hen Hsieh

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Glaciers lost

A winter's tale tackles the rise and fall of ice ages.

Frozen Earth: The Once and Future Story of Ice Ages

by Doug Macdougall

University of California Press: 2004. 267 pp.

\$24.95, £15.95

Euan Nisbet

"Civilisation exists by geological consent." Doug Macdougall's book starts by quoting the American thinker Will Durant. But talented businessmen and managers, even vice-presidents and chancellors of the exchequer, who press our titanic economies to steam ever faster, might ponder some biblical wisdom, from Job 12:8: "Speak to the Earth, and it shall teach thee."

Frozen Earth is a comfortable-to-read ecotale, exactly the right present to keep a liberal granny quiet in the post-Hanukkah, post-Christmas longueurs before the world begins again in 2005. Denied *North by Northwest* again, while the kids soak in neo-reality reloading *The Matrix*, she can lose herself in a palaeo-world by the fireside. Who knows, she might rise up on wings of an eagle and force them to watch *The Day After Tomorrow*.

Ice is a geological rock, yet it passes through the atmosphere. Strangely, it floats on its own liquid. Stranger yet, for billions of years on our planet, the solid, liquid and vapour of water have all coexisted and exchanged, while hydroxyl, the daughter of water, rules the chemistry of the skies above. What controls the ice? Why does it come and go? In *Paradise Lost* (book 10, line 668), John Milton suggested that the great impact that tilted the planet was God's response to the apple-biting crime by the first Iraqi dictator: "Some say He bid his Angels turn askance / The Poles of Earth twice ten degrees and more / From the Sun's axle; they with labour push'd / Oblique the Centric Globe." It's the wobbles and eccentricities on the axle that start and end ice ages.

Macdougall's writing is easy, pegging narrative to history of discovery. The story gets going near the salt mine of Bex, Switzerland. Here Louis Agassiz and his family spent the summer of 1836 with Jean de Charpentier and Ignatz Venetz (Macdougall resuscitates many forgotten worthies). The wine must have been good, the culture high and the geology fun. In the warmth of Bex, Agassiz's friends showed him evidence of past glaciation. Once he could read the signs, Agassiz realized that not only Switzerland but half of Europe and North America had been the empire of ice. Even Darwin beat his brow when he realized he had been walking over the evidence without seeing it.

After Agassiz, Macdougall comes under



Season's greetings: a nineteenth-century Christmas card shows the evidence of our own ice age.

the spell of the eccentric genius William Buckland, whose death has been ascribed to eating the preserved heart of Louis XIV. Next comes the long-forgotten Scottish school caretaker James Croll, who suggested the astronomical theory of ice ages. Macdougall also turns to Harlan Bretz, who realized that the 'channelled scablands' of the US Pacific Northwest had been created by the catastrophic drainage of a great lake. Among the largest post-glacial floods, adding more than a foot to the sea level, was the sudden drainage in Canada, some 8,500 years ago, of ice-dammed Lake Agassiz.

Then Macdougall tackles the orbital gyrations of Milutin Milankovitch, which explain the cyclicity of ice ages in terms of changes in the Earth's orbit and tilt. This is the ruling theory, although it is still much disputed. Maybe Milton was right: did the ice-making cycles drive humanity out of a pastoral Eden and into urban living? Then we digress to the far Precambrian, to Snowball Earth, when the entire planet froze over.

Geology tells us that climate can change very quickly. Macdougall describes extraordinary recent discoveries made by studying cores in ice and in the oceans. This is standard reporting, but he gives deserved credit to Lonnie Thompson and Ellen Mosley-Thompson's heroic drive to archive the tropical ice record. So much is ablating, so fast: Kilimanjaro's ice, the diamond in Africa's navel, could be gone in a few years. With satisfying frissons of self-blame over its loss, we prefer angst and weeping to studying or even saving it. We do shamefully little. But the main record is in the Arctic, Antarctic and

ocean cores, and — to voice one slight criticism — there could be more in the book on the astonishing step changes in global climate between 10,000 and 15,000 years ago, and on the superb analytical work now under way.

Macdougall points out that we are still in an ice age, albeit in a warm interval — or at least, we were until the twentieth century. The evidence now suggests that we have ended it. Geology is about climate, but it also gave us the oil and gas that we used to reset the climate.

Now for a rant, where Macdougall, ever-easy, treads but lightly. The hydrocarbon gift is limited and already half-used. How we manage the run-out of oil will have profound consequences. Judged by change in carbon eruction since 1990, very few nations have done well. In terms of the growth in per capita emissions since 1990, there is little difference between the United States and many of the European nations that sling mud at it.

All neglect the air. China looms, as does India. Not only have their energy demands soared with the welcome retreat of poverty, but when they clean up their filthy sulphate-laden air, as they soon must, a major global coolant will vanish.

Who cares about carbon dioxide and methane? Not humanity: the playwright Aeschylus declared the air to be "heaven's protectorate". He discovered his error the hard way. The aeschelonian albedo changed as he aged, gaining a shiny top. But while most bald men suffer only sunburn and mosquitoes, Aeschylus was, to use Pentagon-speak, taken out by a precision-targeted



On the record

Fish were the inspiration for Ray Troll's witty artworks, which adorned T-shirts in the 1980s. This fascination has taken him deeper into the marine world and its evolution, as shown by the detail of spawning ammonites shown

here. Troll is now a celebrity among palaeontologists, and his art has moved from T-shirts to museum exhibitions. *Rapture of the Deep: The Art of Ray Troll* (University of California Press, \$29.95, £18.95) showcases his work.

air strike. A passing eagle, clutching a tortoise, had seen his head. The best way to open a tortoise, if you are an eagle, is to drop it on a clean shiny surface. Exit the father of tragedy, in the only known case of killing two chelonians with one bird.

Though we can set the climate as we choose, we need to guard the bald top of the world. If you don't want to be struck from the air by an unknown unknown, give this book to a campaigning granny. ■

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Ring theory

The Science of Middle-Earth

by Henry Gee

Cold Spring Press: 2004. 256 pp. \$14. To be published in the UK by Souvenir Press.

Michael A. Goldman

We're living in a society where the average person's knowledge of science is so fragmentary that much of what we take for granted looks a lot like magic. Although it starts out as a well-informed critique of J. R. R. Tolkien's work, Henry Gee's *The Science of Middle-Earth* rapidly moves into a lively collection of science essays united by references to life in Middle-Earth, an exploration of the depths of Tolkien's keen imagination. Tolkien's work had a cult following that escalated with Peter Jackson's stunning films of *The Lord of the Rings*. Gee, an editor of *Nature* and a disciple of Tolkien's classic, is equally at home in the modern research laboratory and in the lands of Middle-Earth.

Gee's attempt to entertain and educate about science using Tolkien's fantasy to capture the public's attention may be just what the MTV generation needs. The tour takes us through ecology, evolutionary theory, metabolic-rate physiology, the scaling of body size, biochemistry and genomics.

How about a chainmail vest that can deflect the sharp blow of an orc? In the analysis that follows, the reader learns about pliability, hardness, brittleness and newly conceived materials yet to be synthesized. How do orcs reproduce? Were they cloned or manufactured? Were they fallen humans, or devolved elves?

Far from mere legend, a dragon is a challenging problem in developmental biology. With four legs and two wings, dragons are an ideal opportunity to explain homeotic mutations. Fire-breathing dragons, the only respectable kind, no doubt do a little chemistry to create something like diethyl ether, which, slightly warmed, would probably ignite on contact with air. Excess vapours would burn off slowly as the dragon fumed between rampages.

The denizens of Middle-Earth have legends and a past of their own. Some societies may have devolved over time, losing their best technology. Readers of *Nature* are now familiar with *Homo floresiensis*, known as the Hobbit, who shared Indonesian islands with humans just 18,000 years ago, and maybe more recently. They might represent a devolved form of *Homo erectus*. Gee tells us that our fairy tales could be poorly preserved accounts of events and technologies we no longer remember or understand. Similarly, "the slow erosion of information into repetitive idiocy resonates with what we know of the evolution of the human genome... time

and chance have made nonsensical drifts of huge swaths of once useful genetic information." Wouldn't mobile phones look like magic to someone from 50 years ago, and wouldn't we think it magic if they worked as they should? As Arthur C. Clarke pointed out, any sufficiently advanced technology is indistinguishable from magic.

Gee is alarmed at what he sees as a decline of science as a college major. While we lament the lack of interest in science, we too often worry about it in terms of the profitable innovations science gives us, rather than the simple pleasure of knowing. We expect people to take certain views of the world as law, discouraging questioning, and asking people to take our word for it. "This aggressive promotion of science is flawed, not least because many of its provisions" are "antithetical to the pursuit of science". No matter how fundamental we think a concept is, we need to explain it patiently, rather than insisting the public take it as given. Gee chides evolutionary biologist Richard Dawkins, saying that "it is a tactical error for those promoting science to impose their view on others, for fear of replacing one kind of religion with another, diminishing the very subversive, questioning quality that is the foundation of science."

Touring Tolkien's fantasy world, he asks with the curiosity of a child: "How does this work?" Aside from the fact that the world isn't real, the approach is fully analogous to the science he is trying to portray. He proposes explanations, brings in relevant data, discards a hypothesis and moves on to another. Still, the One Ring remains unexplained, after several laborious pages. "To a scientist, the existence of the inexplicable is a challenge, and a reminder that science always

has more to achieve." More than any morsel of fact we'll take home, we stand to gain from Gee's piercing observations about science education for the third millennium. ■

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Smart underwear for time travellers

How to Clone the Perfect Blonde: Using Science to Make Your Wildest Dreams Come True

by Sue Nelson & Richard Hollingham
Quirk Books/Ebury Press: 2004. 272 pp.
\$16.95/£7.99

Paul Davies

The challenge facing authors addressing politically sensitive issues such as stem-cell research, cloning and robotics lies less with the technicalities and more with the emotion and prejudice surrounding these subjects. Cloning especially revolts many people who lack the foggiest idea of what is actually involved. The authors of *How to Clone the Perfect Blonde* manage to tackle this topic head-on and non-judgmentally by adopting a mixture of fast-paced exposition and gentle humour. That they manage to do this without either patronizing the reader or trivializing the subject is a tribute to their skill. And the formula works equally well with speculative and popular, but technically difficult, topics, such as quantum teleportation and time travel.

Teleportation technology has leapt from the screenplay of *Star Trek*, where it was motivated primarily by the need for a cheap special effect, to the real world of quantum engineering. One of the key properties of quantum particles such as photons is that they may be put into an 'entangled' state. Two entangled photons, even when far apart, remain linked by what Einstein called "spooky action-at-a-distance". Although entangled states cannot be used to send information faster than light, they can be used to reconstruct replicas-at-a-distance. So far, this has been restricted to single particle states a kilometre or so apart, but that has not stopped some enthusiasts imagining scanning a human body, atom by atom, and reconstructing it on Mars, say. Despite the wackiness of this notion, the physics of quantum information is a hot topic, bearing on practical developments such as quantum cryptography and the race to build a code-busting quantum computer.

And therein lies the value of this book. By addressing a wild and engaging speculation, the authors use it as a peg to cover much valuable science. In the chapter on time travel

we learn the basics of the theory of relativity and some of its more advanced ramifications, such as black holes and wormholes. No matter that unrestricted time travel may be a pipe dream, it's a fun topic that we can all identify with, and it involves some interesting mainstream physics and astronomy that might seem dull in a more prosaic context.

My favourite fantasy, which comes near the end of the book, is the idea of uploading the contents of my brain on to a super-computer, to serve both as a back-up in case something horrible happens to the original and as a gateway to a universe of simulated reality, offering potentially limitless fun. Although technically challenging, to say the least, it is hard to see any obstacles of principle to this procedure, and it raises the unsettling question of how I can be sure that the reality I experience is the 'real' reality or just a simulation. Or indeed, whether there is any meaningful distinction between them.

The reader taking a random walk through this speculative playground will learn some surprising facts to help with future quiz nights. I was particularly intrigued to learn about a slime mould able to find its way through a maze, and the disembodied lamprey brain stem that can control a remote robot by responding to light signals. First prize for inventiveness, however, must go to the smart underwear designed to control the temperature of the room the wearer is in.

As the saying goes, there is something for everybody here, not least for scientists at the sharp end of research, who may be in danger of taking themselves too seriously. ■

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Swimming beneath solid seas

Under Antarctic Ice: The Photographs of Norbert Wu

by Norbert Wu & Jim Mastro
University of California Press: 2004. 176 pp.
£26.95, \$39.95

Frozen Oceans

by David Thomas
Natural History Museum Publishing: 2004.
224 pp. £22

Lloyd Peck

The frozen ends of our planet are among the most captivating and least known places on Earth. What takes them beyond our comprehension is ice. The Antarctic ice sheet is kilometres thick and covers an area bigger than Europe. Ice also means that much of the polar oceans are beyond our experience: some 15–20 million square kilometres of the oceans are ice-bound. This environment drives ocean currents, reflects large amounts of heat from the Sun, and is party to the greatest seasonal changes of any seas.

Possibly the most bizarre aspect of the pack ice in the polar seas is the life that inhabits its under-surfaces, and the communities that depend on the productivity of this system. Algae are eaten by protists and invertebrates, such as amphipods and krill; at the top of the food web, fish make their lives on the frozen ledges and tunnels in the ice. Even some of the top predators, such as whales and seals, depend on the productivity of the sea ice and the ice edge. Climate-change predictions suggest that this is a threatened landscape, with both Arctic and Antarctic sea

Star performers: seastars such as *Odontaster validus*, seen here attacking the larger *Acodontaster conspicuus*, thrive in Antarctic waters where anchor ice scours the sea bed.



UNIV. CALIFORNIA PRESS

Skeleton key

R. McNeill Alexander is renowned for his biomechanics textbooks, but in *Human Bones* (Pi Press, \$37.50) he provides a tour of the 213 bones of the human skeleton for a general audience. He explains how our skeletons form and function, and how knowledge of individual differences assists forensics and archaeology. Accompanying the text are stunning photographs, like this one of a Chinese woman's bound foot, taken by Aaron Diskin.



ice due to decline in coming years. Perhaps we should find out as much as possible about this environment while we can. Two new books offer some excellent views and useful information for those interested, from tourists to the completely focused specialist.

Norbert Wu's *Under Antarctic Ice* is crammed with images that I wish I had taken, many of which will inspire feelings of awe and disbelief in those unfamiliar with Antarctic marine biology. The pictures of anchor ice growing from the seabed are particularly different from previous books of Antarctic photos. There is also a fair bit of information at the front of the book that will be helpful to students from school to undergraduate level. My main concern is that the book falls a little between being a textbook and a coffee-table book. However, many people will take sheer pleasure from the pictures and not quibble about the balance between subjects.

David Thomas's infatuation with sea ice and the life associated with it drives him to work long and unusual hours. His book *Frozen Oceans* deals with pack ice: how it forms, the organisms living inside and under it, and how to study it. As with Wu's book, it suffers a little from being somewhere between a textbook and a book for lay readers with an interest in how cold oceans work. I would have liked to see more references to key and current literature, to make it more valuable to students, but the glossary and list of websites will be valuable. This will prove useful background reading for graduate students and scientists moving into polar marine science. It could also be valuable to tourists visiting the polar oceans. The descriptions of the biological communities living under the pack ice are very good, and I learnt something new about the volatile gases, including bromoform and methyl bromide, that are released from algae at the lower surfaces of sea ice. The book is clear and well written, and I particularly liked the descriptions of porridge, pancake and grease ice. ■

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Swine fever

The Whole Hog: Exploring the Extraordinary Potential of Pigs

by Lyall Watson

Profile: 2004. 278 pp. £16.99, \$24.95

Temple Grandin

Wild pigs have adapted to live in many parts of the world, from the Arizona desert to the Asian rainforest. The hairless, wrinkly babirusa, which has strange tusks that grow out of the top of its snout, is the one pig species that cannot breed with others, owing to a chromosomal abnormality. The highly social white-lipped peccaries, from South America, spend hours grooming and smearing the stinking product of their scent glands on each other. Lyall Watson's narrative provides a detailed natural history of both the behaviour and the biology of these and other pigs.

The author is intimately involved with the animals he describes. He grew up in Africa and spent his childhood in the bush. He had a Zulu companion who taught him

which snakes are dangerous and which are safe. Watson explains how pigs are more gregarious than the ungulates that graze the plains. The social life of pigs is more like that of primates than cattle or sheep.

Pigs will eat just about anything. Watson maintains that this lack of specialization makes the pig more curious about its environment and less afraid of new things than animals with more specialized diets. In the porcine brain there are greater associative areas in the cortex, which may make pigs more willing to approach new things. In my own work, it was interesting to compare the behaviour of domestic pigs with that of hand-raised antelope. If a paper bag is thrown in the pen, the pigs will instantly approach and explore, whereas the antelope instantly flee. Antelope run away first, and look and explore later.

Watson's aim for *The Whole Hog* is to bring together all the information about wild and domestic pigs. He did a splendid job with the wild pigs, but the section on modern domestic pigs is weak. There are two odd omissions. The section on the pork industry does not discuss the animal-welfare controversies surrounding industrial-scale pig farms. And there is excellent information on the need to preserve the different species of wild pig, but no discussion of the dangers of a few large breeding companies narrowing the genetic diversity of domestic pigs.

At the end of the book, Watson challenges the reader with the question of whether animals can think and have consciousness. My own view, as a person with autism, is that language is not required for thinking, because I think in pictures instead of words. Maybe animals think in associations of sensory-based memories? ■

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More books for Christmas

In *Vanity, Vitality, and Virility: The Science Behind the Products You Love to Buy* (Oxford University Press, £18.99), John Emsley explains the chemistry behind the ingredients of everyday products such as sunscreens, anti-ageing creams, Viagra and disposable nappies.

Len Fisher's *Weighing the Soul: The Evolution of Scientific Beliefs* (Weidenfeld & Nicolson, £12.99) takes a look at why common sense has often proved to be the enemy of good science, at the struggles of scientists to overcome scepticism, and at how to distinguish the brilliant from the bizarre.

The Best American Science and Nature Writing 2004 (Houghton Mifflin, \$14) is edited this year

by Steven Pinker and contains a thought-provoking selection of essays reprinted from US newspapers and magazines, with contributions from Daniel Dennett, Nicholas Wade and Max Tegmark among others.

Michael Hanlon's *The Real Mars* (Constable, £25) is a sumptuously illustrated history of our attempts to explore the red planet, culminating with this year's successes in the form of Mars Express and the Spirit and Opportunity rovers.

In *Ig Nobel Prizes 2: Why Chickens Prefer Beautiful Humans* (Orion, £10.99), Marc Abrahams describes more of the bizarre and obscure areas of scientific research that have been honoured with an Ig Nobel prize.

Warming the world

Greenhouse effect: Fourier's concept of planetary energy balance is still relevant today.

Raymond T. Pierrehumbert

Jean-Baptiste Joseph Fourier is generally credited with the discovery of the greenhouse effect, whereby the presence of an atmosphere acts to increase a planet's surface temperature. Written in 1827, nearly three-quarters of a century before science advanced to the point where Arrhenius could quantify the phenomenon, how well does Fourier's concept measure up against our current understanding of the greenhouse effect?

First, it is important to recognize what Fourier did not do in his 1827 essay. He did not say that the operation of the atmosphere is analogous to that of a greenhouse — the French word *serre* (greenhouse) does not appear anywhere in the essay — so he should not be blamed for the well known shortcomings of the analogy. Neither did he write down any equations describing the greenhouse effect, nor compute any estimate of planetary temperature.

"In the present work, I have set myself another goal, that of calling attention to one of the greatest objects of natural philosophy," Fourier writes, referring to the problem of planetary temperatures. Thus, the main contribution of the article is the introduction of planetary temperature as a proper object of study in physics. Fourier established the framework of energy balance still in use today: a planet obtains energy at a certain rate from various sources, and warms up until it loses heat at the same rate. Fourier correctly deduced that a planet loses heat almost exclusively by infrared radiation (*"chaleur obscure"* or 'dark heat') and can do so in a vacuum. Infrared had been discovered by Frederick Herschel only 25 years earlier, and the study of its properties occupied much of the attention of nineteenth-century physicists, including Fourier himself — the long gestation culminated in the birth of quantum theory at the dawn of the twentieth century.

Concerning the Earth's heat source, Fourier first made use of his earlier work on heat diffusion to correctly deduce that the internal heat remaining from the formation of the Earth no longer has a significant influence on surface temperature. He recognized that sunlight carries heat, that the atmosphere is essentially transparent to sunlight, that the light is converted to infrared on being absorbed by the surface, and that the atmosphere is relatively opaque to the infrared that

serves to carry the received heat away to space. In consequence, Fourier reasoned, the temperature has to increase (compared with the no-atmosphere case) to allow sufficient infrared radiation to bring the heat budget into balance. Fourier knew that infrared flux increases with temperature, but had no notion of the form of the increase. Another fifty years were to pass before the discovery of the crucial Stefan–Boltzmann fourth-power law.

Recognizing the inadequate state of infrared theory, Fourier turned to an experi-

black-body radiation law, other phenomena not understood at Fourier's time include the role of convection in causing atmospheric temperature to decrease with height, the importance of this decrease in reducing the mean temperature at which the planet radiates to space, the role of minor atmospheric constituents (notably carbon dioxide and water vapour) in determining the infrared opacity, quantum theory relating to infrared absorption and emission, the dynamic nature of water vapour and its consequent radiative feedback, and both optical and microphysical properties of clouds. Fourier's essay set the agenda for much of this work. Inadequate understanding of vertical temperature gradient, water vapour and clouds continues to plague our theories of climate.

Just as important as what Fourier got right is what he got spectacularly wrong. Fourier believed that the Earth receives a significant amount of heat directly from interplanetary space, which he supposed to have a temperature comparable to that of the polar winter. The idea is not in itself preposterous, but what is remarkable is that, in coming to this conclusion, Fourier dismissed without cause alternative explanations he knew about, and indeed refers to in the same essay: thermal inertia and atmosphere–ocean heat transport, which keep the poles and the night warm without any need to invoke an influx of heat from interplanetary space. Fourier's problem was that he fell in love with an idea, and was thus blinded to things he knew. An object lesson for today?

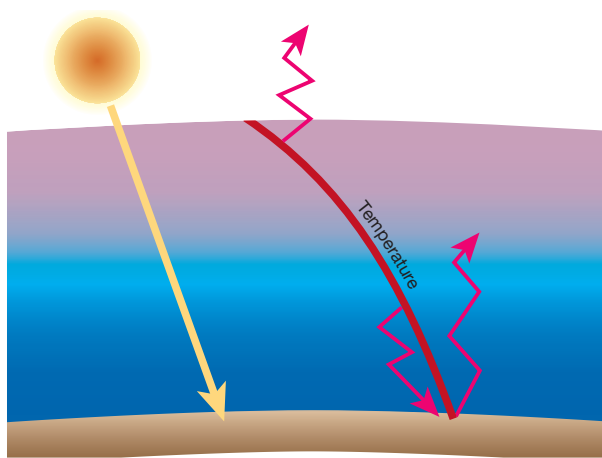
What will future generations think of our present fumbling attempts to understand climate and predict its future course? I myself am left with a disconcerting feeling that some future *Nature* essayist may look back and wonder how we managed to ignore so much evidence that the Earth's climate can change more dramatically and catastrophically than our present models predict. ■

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FURTHER READING

Fourier, J.-B. J. *Mémoires de l'Académie Royale des Sciences de l'Institut de France VII*, 570–604 (1827); a translation of this essay accompanies this article on *Nature's* website.

Bard, E. C. R. *Geosci.* **336**, 603–638 (2004).



Heat is lost by infrared radiation (pink arrows), but some is absorbed by the atmosphere, raising the temperature until output matches input.

ment by the geologist Horace Bénédicte de Saussure. The apparatus for de Saussure's experiment consisted of an insulated box lined with black cork, to which sunlight is admitted at the top through one or more sheets of clear glass. He found that on exposure to sunlight, the interior temperature of the box is greatly elevated, as compared with that found when the glass is removed. De Saussure had built his apparatus as a means of measuring the intensity of solar radiation, but Fourier recognized the implications of the results for the problem of planetary temperatures, in that glass — like the atmosphere — is transparent to sunlight but opaque to infrared. In his discussion of the device, Fourier shows a thorough understanding of the extraneous effects at play, and makes quite clear that it is only the part of the interior warming due to infrared effects that is relevant to the Earth.

Fourier got the essence of the greenhouse effect right — the principle of energy balance and the asymmetric effect of the atmosphere on incoming light versus outgoing infrared. The remaining physics took almost two more centuries to sort out, and the job is still not yet done. As well as the Stefan–Boltzmann

Fowl sequence

Jeremy Schmutz and Jane Grimwood

Chickens have been an invaluable model organism for decades. Their usefulness in research, from genomics to breeding, will further increase with the sequencing of the genome of one chicken species.

The article on page 695 of this issue¹ describes the draft sequencing and initial analysis of the genome of *Gallus gallus* — more commonly known as the red jungle fowl, the predecessor of the domestic chicken, and a valuable experimental organism (Box 1). Describing the first avian genome to be sequenced, this paper and the two that accompany it^{2,3} provide a valuable resource for a diverse set of scientists studying a diverse set of scientific problems. Those who will benefit include agricultural researchers attempting to breed the most productive strain by recognizing links between DNA sequences and attributes such as egg production; comparative genomicists desiring to accurately identify the functional elements of the human genome; and genome-sequence producers, who continue to debate the most effective way of sequencing a vertebrate's large genome.

The draft chicken genome sequence, as reported by the International Chicken Genome Sequencing Consortium¹, has several features that distinguish it from the sequenced mammalian genomes — those of humans, mice, rats and dogs. Weighing in at about 1 billion DNA base pairs, the chicken genome is broken down into 1 pair of sex chromosomes (Z and W, with females being ZW and males ZZ) and 38 non-sex chromosomes (autosomes). The autosomes vary

greatly in size, being described as macrochromosomes (large) and microchromosomes (tiny). Microchromosomes, which range from 5 million to 20 million base pairs, are not common in mammals but are abundant in birds and some fish and reptile species. The consortium's analysis of these microchromosomes in chickens indicates that they are easily discernible from macrochromosomes at the sequence level, because of their relatively high levels of guanosine–cytosine (GC) base pairs (compared with adenine–thymine pairs) and relative lack of repetitive sequences.

Another notable difference between the chicken genome and the average mammalian genome is that the chicken sequence is about one-third the size. This is now explained in part by its markedly smaller amount of 'common repeats' (stretches of sequence that occur many times), including a reduction in the number of degraded copies of gene sequences, a simpler structure of large duplications, and fewer duplicated copies of genes overall.

So much for generalities; how does this draft sequence benefit the various 'special interests' groups mentioned above? First, it provides an initial framework for chicken breeders who want to understand how genetic variation influences traits that are important in the production of domestic

chickens, by allowing the traits to be mapped back to precise genomic locations and genes. These groups have traditionally used quantitative trait loci — an estimate of the occurrence rate of a desirable 'continuous' trait in a population — to link the genetics of a strain to that trait. Continuous traits show graded variation and are controlled by more than one gene; in humans, they include height.

With the draft sequence, however — together with the second paper in this issue² — it will be easier to link specific genetic variations with variations in physical traits. In that second paper, the International Chicken Polymorphism Map Consortium describes numerous single-base-pair differences — 2.8 million of them, in fact — between three lines of domestic chicken (broiler, layer and Silkie) and the red jungle fowl. The map they have developed should allow researchers to identify the genes, and the combinations of gene variations, that produce desirable traits in chicken breeding populations. It should also increase the odds of optimizing a particular trait in subsequent generations.

For some time now, researchers in comparative genomics who are studying the human genome have also been craving the genome sequence of a species in the chicken's rough evolu-

Box 1 Chickens in the lab

Chickens have become one of the predominant model species for biological research, for numerous reasons.

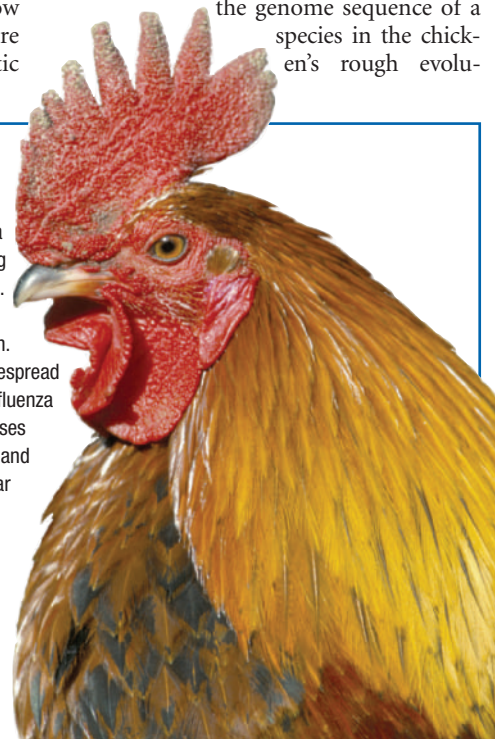
- They are ideal for classical genetics. Chickens are easy to maintain, reproduce rapidly, have large brood sizes and have distinguishable characteristics, making it possible to track traits from parent to offspring.
- Many natural mutant variants exist. Over the past 60 years, chickens displaying extremes in heritable characteristics have been maintained in breeding populations, allowing researchers to investigate the underlying genetic mutations.

- They are ideal for studying vertebrate development. Chicken embryos develop in morphologically similar ways to mammals, yet, unlike mammalian embryos, are accessible to study (in the egg). Much of what we know about human limb formation has been uncovered through studies of chickens.
- They provide a model of human genetic diseases. Chicken lines have been created that show identical symptoms to those of patients with common debilitating diseases, including muscular dystrophy, epileptic seizures and decreased immunological responses. Studying these diseases

in chickens leads to a greater understanding of the genetic causes.

- They allow the study of viral infection. There have been widespread outbreaks of avian influenza and other animal viruses in humans. Chickens and mammals have similar immunological responses, allowing researchers to study the mechanisms of infection and the genetic basis of susceptibility.

J.S. & J.G.



JORG & PETRA WEGNER/BRUCE COLEMAN

tionary position. In general, researchers have a good grasp of how to identify those portions of a genome that are translated into proteins, by aligning sequences of messenger RNAs, the precursors of proteins, against genomic sequences of interest. One can also identify these 'coding' genomic sequences by comparing the DNA of organisms that are evolutionarily distant. For example, stretches of sequence that have been preserved in humans and fruitflies are likely to be very important for the functioning of the organisms. These sequence stretches are called conserved elements.

However, now that the human genome sequence is essentially finished⁴, researchers would like to do more than just identify the sequences that are translated into proteins. They also want to understand all of the regulatory structures present in a genome — structures that might, for instance, adjust the amount of protein manufactured from a particular gene. These structures are collectively known as functional elements, and the chicken, having diverged from humans more than 310 million years ago, is considered the best example so far of an 'outgroup' with which to identify them. Because enough differences between the human and chicken sequences have accumulated over this period, one can zero in on the precise base pairs that evolution has left alone for all these years — the base pairs most likely to be functional in the human genome. By comparison, the mouse, which split from humans only 75 million years ago, is too similar at the base-pair level, leading to difficulties in identifying functional elements⁵.

The consortium's initial analysis¹ describes 70 million base pairs of sequence that are highly conserved between chickens and humans. This includes base pairs within genes, but also base pairs that are between genes and therefore relate to potential functional elements (interestingly, many of these seem to be at a considerable distance from genes). Questions surrounding what these structures are and why evolution has constrained them over time will only be answered with targeted experiments, some of which are beginning to get under way⁶.

Finally, for those who concentrate on generating large-scale genomic sequences and resources, the chicken genome represents another in a series of grand experiments to balance two different approaches. Traditional clone-by-clone approaches (see, for example, refs 4, 7) — which involve cloning a genome into bacterial artificial chromosomes (BACs), mapping the clones, then sequencing them and assembling the sequences by using the map — are time-consuming but generally produce an accurate representation of all regions of the genome. Whole-genome shotgun⁸ (WGS) is quicker, because it involves shattering the whole genome into pieces, sequencing the

fragments and assembling them by computer, but it often fails to represent all regions accurately.

The chicken sequence presented here is a halfway house: it is not a straight WGS assembly, but has been revised according to a physical map of 180,000 BAC clones, detailed by Wallis *et al.*³ on page 761. This map was crucial in ordering and localizing the sequence pieces generated by WGS. Thus the assembly captures an impressive 98% of the sequence over most of the genome, with that number falling slightly in very GC-rich regions. The authors were also able to locate partial or complete sequences of at least 97% of coding genes that were previously known to exist.

However, the genome has received no directed 'finishing' work, and issues do still exist — there is a distinct lack of continuity in 10% of the gene-rich regions, and there are perhaps 1.4 million base pairs of sequence that are in the wrong position. Recent studies⁹ suggest that, even with algorithmic improvements, WGS assemblies fail to resolve large-scale duplications in vertebrate genomes; even with a BAC map, recently duplicated

sequences in the chicken assembly are poorly resolved¹. And the authors suggest that one reason why they were able to resolve most of the WGS sequence was the minimal repetitive content of the chicken genome, so the experience will not necessarily translate to all vertebrate genomes. As we move forward in this post-genomic era, we must learn from all past experience, so that we can maintain the high quality we have come to expect from genome-sequencing projects. ■

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Microbiology

Jekyll or hide?

George A. O'Toole

Many bacteria can adopt different lifestyles: in a free-living state, they are virulent and cause disease; in a surface-attached community, they are less virulent but may go unnoticed. How is this 'decision' made?

In the November issue of *Developmental Cell*, Goodman and colleagues¹ report the identification of a regulatory system in the bacterium *Pseudomonas aeruginosa* that determines whether it causes disease or lies low and simply persists. This bacterium is of interest to the medical community because of its ability to infect people whose immune system is damaged, who have sustained serious burns or an eye injury, or who suffer from cystic fibrosis. Goodman *et al.* found that inactivating a so-called two-component regulatory system in *P. aeruginosa* results in a strain with a markedly decreased ability to cause disease, but an increased ability to form surface-attached, persistent communities known as biofilms (Fig. 1).

Although there are many variations on bacterial two-component regulatory systems, their basic job is to constantly sample the external environment and transmit this information to the bacterial interior. This allows the organism to adapt to an ever-changing environment. Goodman *et al.*¹ discovered a new protein component of a new such regulatory system, a component that they call RetS.

They also found that a RetS-deficient *P. aeruginosa* strain was better than a wild-type strain at forming a biofilm on both an abiotic surface, namely glass, and a biotic surface, cultured hamster cells. The RetS-deficient bacteria were, however, less able to damage the hamster cells they colonized, and to cause disease in a mouse model of pneumonia. Outside the lab, the ability of *P. aeruginosa* to form biofilms is best known with respect to abiotic surfaces such as catheters, but it might also be able to produce biofilms on tissues within a host, in diseases such as otitis media (earache) and cystic fibrosis². It seems, then, that Goodman *et al.* might have identified a control element that allows this bacterium to switch between a virulent, disease-causing state and a biofilm state in a mammalian host. The biofilm state, although less virulent, might allow the microbe to persist for longer.

To understand better how the protein might control this pathogenesis/persistence switch, the investigators used DNA microarrays to identify all the genes in the organism that are regulated by RetS. They found that, in the RetS-deficient strain, the expression of genes required to make a 'type III secretion

system' — necessary for *P. aeruginosa* to cause a short-term infection — was reduced by as much as 25 times. The expression of other genes associated with virulence was also reduced; these genes include those that produce surface appendages called pili, as well as those that encode toxins such as LipA and ToxA.

These data imply that RetS is normally required for the full expression of the factors required to produce an acute infection, and are consistent with the decreased ability of RetS-deficient *P. aeruginosa* to cause disease. In contrast, *P. aeruginosa* genes that are involved in the formation of the sugar-rich matrix that encloses a biofilm — the *psl* and *pel* genes^{3–6} — were markedly upregulated in the mutant bacteria. This suggests that RetS usually turns off the genes needed to make a biofilm.

We make choices every day on the basis of the information at hand. Bacteria must do so too, and the outcomes of their decisions can have life-or-death consequences, both to the bacteria and to the host. For bacteria, these choices — such as whether or not to form a persistent biofilm — are based in large part on local environmental cues, and are effected through altered gene expression. For instance, *P. aeruginosa* decides to form biofilms on abiotic surfaces, such as catheters or contact lenses, only when an energy source (such as sugars) and other nutrients (such as iron) are readily available^{7,8}. Otherwise, it remains free-living. Goodman and colleagues' findings suggest that *P. aeruginosa* also has a decision to make when in the context of a mammalian host: does it cause a short-term infection or does it persist in a biofilm state? An acute infection provides a means of bacterial propagation, whereas in a biofilm the organism is lying low and is thus less likely to be recognized and attacked by the immune system.

The very existence of a regulatory system that mediates this decision suggests that the choice is a crucial one for microbes, and one that they must constantly re-evaluate. Studies of *Bordetella bronchiseptica* — an organism related to the microbe that causes whooping cough — provided one of the first molecular illustrations of this decision⁹. Thus, *B. bronchiseptica* forms biofilms best when the genes required for acute infection are turned off. However, expression of a toxin required for acute infection can block biofilm formation, hinting that the functions required to cause disease and those required to make a biofilm might actually be incompatible. Similarly, my own group has found that expression of the type III secretion system, required for acute infection in *P. aeruginosa*, also inhibits biofilm formation¹⁰.

We would do well to continue learning about how bacteria can switch from disease to persistence and back again. A better

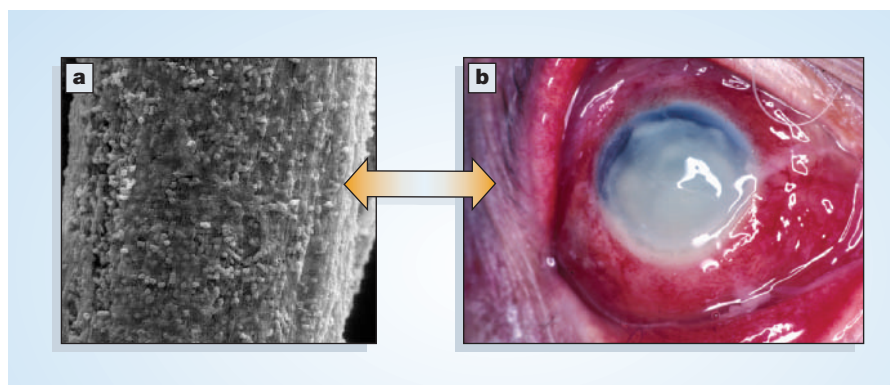


Figure 1 Persistence versus infection. Goodman *et al.*¹ have discovered a regulatory system in the bacterium *Pseudomonas aeruginosa* that might enable it to choose between two lifestyles in a mammalian host: growing as a surface-attached, persistent community (a biofilm), or causing a short-term infection. a, An electron micrograph of a *P. aeruginosa* biofilm on a suture. Individual cells can be seen, surrounded by a sugar-rich matrix. (Courtesy Jon Budzik and the Ripple EM facility, Dartmouth College, New Hampshire.) b, An inflamed eye — the effect of an acute *P. aeruginosa* infection of the cornea. (Courtesy Patrick Saine and Michael E. Zegans, Dartmouth Medical School, New Hampshire.)

understanding of this decision could lead to new strategies for dealing with bacterial infections.

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Planetary science

Volcanoes on Quaoar?

David J. Stevenson

Quaoar, a large body in the Kuiper belt, has crystalline water ice on its surface, yet conditions there should favour amorphous ice. Does this mean that resurfacing has taken place — perhaps even volcanism?

Our planetary system does not end at Pluto. Hundreds of bodies exist in the Kuiper belt, which extends outwards from Pluto, sharing the same plane as the planetary orbits. The largest known bodies in the Kuiper belt are not much smaller than Pluto, and some have similar dynamics to that planet. Although the existence of the Kuiper belt had long been hypothesized, the first Kuiper-belt body was discovered only in 1992, by Jewitt and Luu¹. These same authors now propose² that Quaoar, the largest known of these bodies, has crystalline water ice on its surface and possibly also ammonia (see page 731 of this issue). The presence of crystalline ice is surprising, because it is widely believed that its formation requires a temperature of around 100 K or more — substantially higher than the surface temperature of these bodies. The precise temperature required, however, is not known and may not be the same in laboratory

experiments as it is in space. Yet it might be that we are seeing evidence for 'planetary' processes such as volcanism within these bodies.

The discovery and characterization of the Kuiper belt is among the most important developments in planetary science in the past decade³. As is usual with the discovery of new bodies (inside or outside the Solar System), the initial excitement focused on the dynamical implications: why do they occupy these orbits and how did they form? Some orbital migration may occur, but it is likely that these bodies never experienced much higher surface temperatures than the ambient conditions provided by the Sun (temperatures of about 50 K or less). Quaoar, discovered in 2002 by Trujillo and Brown, is the largest body to be found in our system since the discovery of Pluto in 1930. It has a radius of about 650 km, roughly half that of Pluto. The composition and nature of the

surfaces of these bodies are difficult to determine, yet such characteristics may be important for understanding their history. Colour and brightness (albedo) can be informative, but spectroscopy at near-infrared wavelengths is the preferred technique of investigation.

Water is the most abundant condensed material in the Universe, and it should form the 'bedrock' for solid bodies in the outer Solar System. This does not necessarily mean that the water would be readily observed; it could be hidden beneath a mantle of other material. Still, it is not surprising that Jewitt and Luu² observed the distinctive spectroscopic feature of water ice. But water ice that forms and remains at very low temperatures would be expected to be amorphous — that is, lacking the periodic structure of crystalline water ice. This is because the highly coordinated architecture of a crystal lattice is difficult to establish when molecules are added to a substrate at very low energy (temperature). At higher temperature, amorphous ice rearranges to crystallize into the ordered, thermodynamic ground state (and releases latent heat as it does so).

More controversially, Jewitt and Luu claim evidence for ammonia ice. The slight dip they see in the spectrum of reflected light around a wavelength of 2.22 micrometres is a subtle characteristic, and seems to be part of a broader spectral feature that is imperfectly understood. Brown and Trujillo have observed the crystalline water-ice feature independently (personal communication), and they also report a putative feature at 2.22 micrometres — but they favour methane or some other explanation for the shape of the spectrum in this region. Irrespective of whether one believes the evidence for ammonia — and it would indeed survive for only a limited time on the surface — it is likely that this molecule is present to some extent in the internal make-up of Quaoar: ammonia is present in interstellar space and is a natural (although possibly minor) carrier of nitrogen in the Universe. This raises the intriguing possibility of water-ammonia volcanism.

For any Kuiper-belt object with a radius greater than about 200 km, the time taken for heat to diffuse through the body is longer than the lifetime of long-lived radioactive elements — the same elements that provide most of Earth's geothermal heat flow. These bodies are expected to contain sufficient rocky material, half or more by mass, for their temperature to easily exceed 200 K deep down during their evolution. An ice mixture that contains both water and ammonia would begin to melt at a temperature of about 175 K. The melt produced would be one-third ammonia and two-thirds water, and it would be much less dense than the surrounding ice-rock mixture. It could percolate upwards and perhaps segregate to form a

fluid-filled crack. Even in the low gravity of Quaoar, a crack of a few kilometres in length would create a hydrostatic 'head' exceeding several atmospheres of pressure, probably sufficient to enable the crack to propagate to the surface, or near to it. All of these processes are directly analogous to basaltic volcanism on Earth and other terrestrial planets. The cooling lava would contain crystalline water ice and crystalline ammonium hydrate, which could become exposed by infrequent impacts on the surface of Quaoar.

This is speculative and might be unnecessary. There may be non-thermal processes that make crystalline ice and do not depend on the presence of a massive body with a warm subsurface. Crystalline ice has been observed in the disks around newly forming stars⁴ (but we do not know the thermal

history of this dust). Evidently, more observations are needed, both of our Solar System and of more distant targets. Data from the infrared space observatory Spitzer⁵, currently in orbit, are expected to help. More laboratory data are also in order. But we should not exclude the possibility that planetary processes such as volcanism could occur in volatile-rich bodies at these outer reaches of the Solar System. ■

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Behavioural biology

Name that tune

Daniel Margoliash

Understanding how early auditory memories are laid down could help to explain their role in vocal development. Some ingenious experiments in birds provide fresh ideas about how such memories are represented.

A central discovery that emerged from the early studies of Konrad Lorenz and Nikolaas Tinbergen was that animals have innate predispositions that guide the learning of species-appropriate signals. Bird-songs are a good example of such signals. Young songbirds must learn their specific songs by listening to adults, and research has provided insight into how auditory memories are formed under species-specific constraints, and how those memories help to guide song development. Yet the organization of the 'acquired template' for song learning that is formed from the early experience of hearing other birds sing remains a mystery, at both the neurobiological and (more surprisingly) the behavioural levels. Elsewhere in this issue (page 753), Rose *et al.*¹ describe certain features of that template: they show how white-crowned sparrows (*Zonotrichia leucophrys*, Fig. 1), birds that have long been used in this line of research, can assemble a complete song in proper sequence when exposed early in life only to fragments of that song.

Song in birds (and speech in humans) is the culmination of the interplay between innate physiological constraints and



Figure 1 Model singer — the white-crowned sparrow.

environmental cues, including social interactions, song models and auditory feedback. The process is further shaped by developmental constraints such as the limited 'sensitive periods' when song models can be memorized. Researchers investigating song learning must therefore assess the degree to which a bird's success or failure to acquire a song results from interactions among these many influences. For example, when presented with tape-recorded songs of their

FRANK LANE PICTURE AGENCY/CORBIS

own and other species, juvenile sparrows tend to choose the songs of their own species². But this innate tendency can be overcome if a bird has a live tutor of another species: if the song structure falls within the capabilities of the sparrows, they can learn to sing the alien songs³.

In general, the morphological structure of song segments (phrases) is a surprisingly weak constraint on song acquisition for white-crowned sparrows. For example, although the trill phrase normally consists only of downward-sweeping frequency modulations, birds can learn songs with upward-sweeping modulations^{4,5}. But what about the order in which phrases are sung? Sparrows tutored on only individual phrases assemble a more complex and species-typical pattern but fail to produce a normal song⁶.

In their research, Rose *et al.*¹ found a similar limitation. However, they then went on to show that when sparrows were presented with phrase pairs (for example AB, BC, CD, DE or DE, CD, BC, AB) in fixed order, the birds tended to assemble the song correctly (ABCDE). Remarkably, if birds were trained with BA, CB, DC, ED, they tended to learn the song EDCBA. These results show that, for white-crowned sparrows, the acquired template represents sequence information; that the minimal information for identifying a sequence of appropriate length is sufficient to describe song; and that environmentally supplied sequence information can overcome the innate tendency to start songs with whistles ('A' in the above examples).

How is this information represented in the brain? How are the early memories of songs laid down, and how do they guide vocal learning? Songbirds possess a set of distinct forebrain cell groups ('nuclei') associated with song production, perception and learning — the so-called song system. Early studies of adult birds showed that playback of a bird's own song elicits much stronger responses from song-system neurons than does playback of virtually any other song, a clear result of the learning process. In white-crowned sparrows, some neurons in the nucleus HVC, a site of sensory and motor integration in the song system, exhibited own-song selective responses, but only when a bird was presented with sequences of two phrases drawn in proper order from the bird's song⁷. The individual phrases failed to stimulate such combination-sensitive neurons, whereas most neurons in the HVC responded to individual phrases⁵.

Rose and colleagues' results¹ imply that an acquired template sufficient for normal song learning can be assembled from sequential pairs of phrases. It is tempting to link these two observations: auditory memories are laid down (in part) as sequences that are represented by combination-sensitive neurons in the HVC. This also requires that such neurons have special 'status' as

auditory memory neurons (for example, they could be disproportionately common as HVC output neurons), or strongly influence the discharge of the more numerous HVC neurons that respond to single phrases. One caveat is that the observed responses of own-song-selective neurons, recorded in adults, could be the result of establishing early auditory memories, but could also result from practice during vocal development. Evidence from young zebra finches, constrained to sing abnormal songs, shows that at least some song-system neurons achieve selectivity for auditory memories apparently independently of what the bird is singing⁸.

A related question is whether the acquired template exists as a single group of cells that is modified by early auditory experience, or whether it has a more distributed representation. In the bird forebrain, secondary auditory pathways give rise to at least some of the auditory input to the song system⁹. Recent evidence, again in adult birds, ties specific changes in auditory responses within these pathways to perceptual learning of songs from different individuals¹⁰. If these same pathways are also modified during juvenile acquisition of auditory memories, at the same time that combination-sensitive responses are first specified in the song system, this would be evidence for a distributed representation across multiple cell groups.

Rose and colleagues' studies extend Peter Marler's classic work on white-crowned sparrows that started more than 40 years ago¹¹. They add confidence to the view that the next decade will see dramatic progress in tackling the fundamental physiological questions about the song system. The promise of this system is that research can go beyond addressing which neurons and currents are modified during various phases of learning, to explore how they are arranged into a code for larger units of behaviour and how they produce physiological variation and adaptation. Playing Mother Sparrow to nestlings can be gruelling work, but the new results¹ emphasize that behavioural biology is an essential part of this research programme. ■

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100 YEARS AGO

The sale of Chartley Park, Staffordshire, the hereditary seat of Lord Ferrers, involves also a change of ownership of the remnant of the celebrated herd of white cattle which have been kept there for the last 700 years... It was long considered that the herds of cattle in various British parks were direct descendants of the wild aurochs, but it is now generally admitted (largely owing to the writings of Mr. Lydekker) that they are derived from domesticated albino breeds nearly allied to the Pembroke and other black Welsh strains, some of which show a marked tendency to albinism... The theory advocated by a later writer... that the British park cattle are the descendants of a white sacrificial breed introduced by the Romans rests upon no solid basis. The Chartley cattle, believed to be reduced to nine head, are to be captured by the purchaser — no easy task.

From *Nature* 8 December 1904.

50 YEARS AGO

The Conquest of Plague. This is a comprehensive study of plague. The author, Dr. L. Fabian Hirst... reminds the reader that primitive beliefs and observations may hold truths that are only revealed to the man of science centuries later. Instances of this are the epidemics of plague among the Philistines, described in the Old Testament (I Samuel, v, vi), which are associated with the presence of small rodents. The Hebrew word *akbar* may mean rat or mouse. If the civic authorities of London had read their Bible more carefully, they would not have favoured the rat population in ordering the destruction of cats and dogs as possible vectors of plague in 1665. Dr. Charles Singer has pointed out that during the seventeenth century natural causes were first assigned to plague, and even that it was due to the multiplication of minute organisms, a hypothesis not verified for two hundred years... On the vexed question as to whether Kitasato or Yersin discovered the bacillus of plague at Hong Kong in 1894, Dr. Hirst examines the evidence and states (p. 108): "The conclusion must be that both bacteriologists discovered the plague bacillus within a few days. Kitasato saw it first, but Yersin, the former *preparateur* of the Pasteur Institute, was probably the more accomplished technician and gave by far the best description of the micro-organism".

From *Nature* 11 December 1954.

Climate change

Tropical flip-flop connections

John C. H. Chiang and Athanasios Koutavas

A long climatic record shows that episodic wet periods in northeastern Brazil are linked to distant climate anomalies. The ocean–atmosphere system can evidently undergo rapid and global reorganization.

Every few years, the semi-arid but densely populated region of northeastern Brazil experiences severe drought. In 1997–98, exacerbated by poor distribution of resources, such an event caused great hardship. And in the catastrophic drought of 1877–79, hundreds of thousands of people perished. The causes of these conditions are neither accidental nor local, but part of an orchestrated sequence of large-scale atmospheric and oceanic processes over the entire tropical Atlantic (Fig. 1). These processes lead to changes in the seasonal southward migration of the Intertropical Convergence Zone (ITCZ)¹, the rainfall band that spans the Atlantic. When the southward migration of the ITCZ in February–May stops short of northeastern Brazil, the rainy season fails.

For those seeking knowledge of past climate changes, however, northeastern Brazil presents an opportunity: what better place to focus on than one where flip-flopping climate is singularly representative of changes throughout the entire tropical

Atlantic? Wang *et al.* (page 740 of this issue)² have done just that. They find that over the past 210,000 years northeastern Brazil underwent episodic changes from its usual semi-arid state to a wetter climate, implying a persistently southward-shifted ITCZ. Unlike the present-day disturbances, which last a few years at most, these past wet episodes typically persisted for several centuries. The authors deduced the presence and duration of the wet periods from the growth patterns and ages of mineral deposits, called speleothems and travertines, in the northern part of Bahia state in Brazil.

Speleothems are deposited in caves, and travertines along spring-fed rivers and streams, when calcium carbonate precipitates out of supersaturated ground and spring waters; deposition is a good indicator of abundant rainfall. Northeastern Brazil is usually too arid to support the formation of either type of deposit (as is the case for present-day conditions), but repeated episodes of persistently wetter conditions evidently allowed them to form in the past.

The remains of vegetation embedded in the travertines show that semi-deciduous forest abounded during the wet phases, linking the Amazon rainforest in the northwest with the Atlantic rainforest along the eastern coast of Brazil. An intriguing idea put forward by Wang *et al.* is that the unusual biodiversity of these rainforests can be attributed partly to the floristic exchange made possible by the recurring shifts to a wet climate.

The more remarkable aspect of this study, however, is identification of the apparent synchrony of wet periods in northeastern Brazil with climate changes near and far. The timing of the wet periods can be accurately determined using uranium–thorium dating, a system that relies on decay products of radioactive uranium-238. The ages of speleothems analysed by Wang *et al.* correlate remarkably well with the timing of climate changes in different parts of the Northern Hemisphere — specifically, with weakening of the East Asian summer monsoon; with cold periods over Greenland; and with episodes in the North Atlantic, known as Heinrich events, that are characterized by massive release of icebergs into the open ocean from continental glaciers. Closer to home, Wang and colleagues' results elegantly confirm earlier indications of a southward-displaced ITCZ deduced from mineralogical changes in sediments of the Cariaco Basin, off Venezuela³.

What do these records tell us about the climate system? Cold episodes over the

Plant biochemistry

Green catalytic converter

A key component of the Kyoto Protocol — the international agreement that aims to reduce greenhouse-gas concentrations — is the control of carbon emissions into the atmosphere. This will require industries to reclaim and recycle the carbon dioxide they currently discard. Elsewhere in this issue (see *Nature* 432, 779–782; 2004), Jörg Schwender and colleagues show that plants are way ahead of us in this respect.

Oil is the major storage product in most seeds, but the standard biochemical route for its synthesis was thought to be quite wasteful. Plants employ a variation of glycolysis, the metabolic pathway also used by animals, which breaks down glucose to produce energy. One of its end products is pyruvate, which can be converted to acetyl-CoA, a precursor for fatty acids and oils. This reaction also produces CO₂. For every two carbon atoms

that are made into oil, at least one should be lost as gas.

Schwender *et al.* found that oil production in seeds of *Brassica napus* (oilseed rape or canola; shown here) was nowhere near as inefficient. Using radioactive labelling, they measured the ratio of carbon used to carbon lost as almost three to one. Closer investigation showed that these savings are achieved by a previously unsuspected function of probably the most abundant protein in the world, Rubisco.

Rubisco catalyses a critical reaction in photosynthesis. It combines CO₂ with the sugar ribulose 1,5-bisphosphate to produce two molecules of phosphoglyceric acid as part of a complicated series of chemical reactions known as the Calvin cycle. It turns out that rape seeds have high levels of Rubisco, but no detectable Calvin-cycle activity. Instead, the enzyme's supply of



ribulose 1,5-bisphosphate is synthesized afresh from fructose and glyceraldehydes, allowing it to soak up the CO₂ produced from making acetyl-CoA. The resulting pyruvate is then used to make more oil.

Not all seeds can reclaim CO₂ in this way; sunflower seeds, for example, are much less efficient in

their oil production. The difference is that growing *Brassica* seeds are green. Despite being enclosed in a pod, enough light filters through to be captured by the pigment chlorophyll, supplying the energy needed for the synthesis of ribulose. In seeds, at least, it is only the greens that recycle. **Christopher Surridge**

North Atlantic during the last glacial period are thought to have arisen from abrupt variations in the Atlantic's thermohaline circulation⁴, a density-driven circulation that carries warm and salty surface waters to the northern North Atlantic. As these waters release their heat to the atmosphere they become cold and dense, then sink, and return south as a deep current. A weakening or shutdown of this circulation — achieved by freshening the ocean surface waters from glacial meltwater or through other means — reduces the heat transported to the North Atlantic and cools the climate there. These changes in thermohaline circulation are in essence high-latitude processes with no obvious link to the tropics; but recent discoveries have documented the presence of abrupt events in the northern tropical Atlantic, Pacific⁵ and Indian Ocean⁶ regions. And Wang *et al.* now establish their existence in the southern tropics with a dating accuracy that puts the synchrony with events in the North Atlantic, and in other tropical locations, beyond reasonable doubt.

This development significantly modifies our view of abrupt climate change. Shifts in the strength and distribution of atmospheric convection in the tropics dramatically affect the global climate by altering the global atmospheric circulation — analogous to how the El Niño–Southern Oscillation system in the tropical Pacific affects global climate today⁷. The transport of water vapour will change as a result, possibly affecting the salinity and hence the density of the North Atlantic surface waters that determines the strength of the thermohaline circulation⁸. Taken to the logical extreme, a competing 'tropical driver' hypothesis for abrupt climate changes could easily explain the observed global synchronization — although, as yet, there is no convincing model for how such a driver might operate⁹.

It is nonetheless clear that a complete explanation of abrupt climate change must incorporate both the thermohaline circulation in the North Atlantic and climate processes that occur in the tropics. Results from simulations with climate models offer tantalizing clues as to how this might come about (see ref. 10 for an example). When the thermohaline circulation is forced to shut down, the models respond with rapid and widespread cooling of climate in the Northern Hemisphere and rearrangement of tropical rainfall. The Atlantic ITCZ seems to be particularly sensitive in this regard, and shifts in a manner consistent with the data of Wang and colleagues. Although it is still not clear how the global connections are set up, the tropical Atlantic may have a pivotal role in linking the high latitudes to the tropics^{11,12}.

So, what lies ahead? We can hope for rapid progress in characterizing the timing and spatial extent of abrupt climate changes, particularly in the tropics and Southern

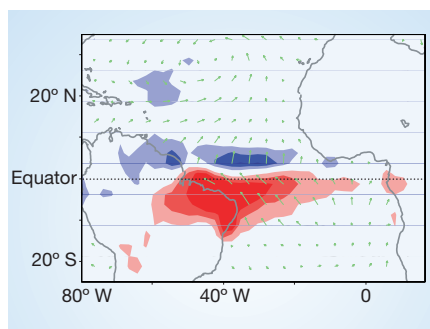


Figure 1 Determinants of present-day drought years in northeastern Brazil. An anomalous north–south gradient in ocean surface temperatures near the Equator (not shown), with warmer conditions in the north, drives a low-level atmospheric circulation, shown by arrows, that strengthens the trade winds in the southern Atlantic and weakens them in the north. The result is to impede the southward migration of the Atlantic Intertropical Convergence Zone, which increases rainfall in the northern tropical Atlantic (blue) and reduces it in the southern tropical Atlantic and northeastern Brazil (red). The opposite set of conditions, persisting over several centuries, is thought to characterize the wet episodes identified by Wang *et al.*².

Hemisphere where data remain sparse. In this regard, the future looks bright for studies of speleothems, given the outstanding dating accuracy and time resolution that they offer. But speleothems are, of course, confined to land: marine records lack their advantages but remain essential.

For modelling, perhaps the key lesson offered by Wang *et al.*² is a deeper appreciation of global interconnections in climate change. This view resonates with basic

notions of how climate adjusts to forcings to maintain energy balance, necessitating changes to the Equator-to-pole temperature gradients and global transports of energy and moisture. But the palaeoclimate records are helping to provide an increasingly detailed view of how this system operates in the real world. Our knowledge of the dynamical adjustments to climate change and their sensitivities across models is still developing, and there is a substantial gap between model simulations and the intricacies of climate changes inferred from palaeodata. Bridging that gap is crucial to reaching the stage where we can make confident predictions about the consequences of our own, self-imposed climate change. ■

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Hearing

Channel at the hair's end

Jonathan Ashmore

Ion channels controlled by sound underlie the sense of hearing. Having long eluded researchers, the first such mammalian channel has now been identified in the mouse inner ear.

When you hear a sound or shake your head, the hair cells of your inner ear convert movement into the electrical currency that the brain understands. But how such transduction from mechanical to electrical signals works on a molecular scale remains unclear. The problem is that the ear's hair cells are relatively few in number, and the molecules involved are sparse, so progress has been slow. On page 723 of this issue, however, Corey and colleagues¹ suggest that a key component of the vertebrate 'mechano-transducer' is an ion channel called TRPA1.

Sound waves or head movements deflect rod-like projections called stereocilia on hair cells. These stereocilia stand out a few micrometres from the cell surface, and when they are rocked, their relative sliding motion pulls on a series of extracellular linking proteins that run between their tips — the 'tip links' (Fig. 1a, overleaf). The pulling makes ion channels in the stereocilia open, causing the hair cells to send signals to the brain. The identity and precise mechanism of action of these mechano-transducing channels remain obscure, however.

There have been hints from studies of

non-mammalian species that the transducers might belong to the TRP superfamily of ion channels. Initially described in fruit-flies (*Drosophila*), this diverse family of membrane proteins now boasts members throughout the animal kingdom that are implicated in intracellular calcium release, osmotic regulation, temperature sensitivity and pain mechanisms^{2,3}.

When TRP-family members were first found in specialized mechanosensory cells of *Drosophila* and zebrafish, they caused considerable excitement. The mechanosensitive bristles of *Drosophila*, for example, involve a TRP channel protein called NOMPC (alternatively named TRPN1)⁴, and a second TRP channel, Nanchung, is found in the fly's hearing organ⁵. The structure of NOMPC suggested that it was a good candidate for a mechanosensory channel in other organisms, but it turned out to have no direct counterpart in most vertebrate genomes, and the search for the vertebrate transducer faltered.

Corey *et al.*¹ now argue that at least one component of the vertebrate mechano-transducer is indeed another member of the TRP superfamily, TRPA1. Described only recently, TRPA1 (also known as ANKTM1)⁶ is a close relative of NOMPC. It is found in a subset of neurons, and is associated with sensing painful cold stimuli, being activated just below room temperature. It is also activated by the contents of kitchen cupboards (such as mustard oil and cinnamon) and bathroom cabinets (including icilin from cold creams), and, even more tantalizingly, by cannabinoids⁷.

Corey *et al.* make their case using several lines of argument. Indeed, there is a tendency in this field to use a variety of species in a single report, and the authors studied hair cells in the hearing and balance organs of mice, but derived corroborating details from the larger cells of frogs and from genetic manipulation of zebrafish. By exhaustively screening all 33 mouse TRP channels, Corey *et al.* found that TRPA1 is expressed in hair cells only when the cells become functional mechano-transducers. When the gene encoding TRPA1 is shut down, mechano-transduction is reduced, as detected either by electrophysiological measurements or by dye permeation through the channel. Moreover, turning off the zebrafish relative of the TRPA1 gene produces hair cells that do not work well.

It seems unlikely that the hair-cell transducer channel is made up of only TRPA1 protein subunits. TRP channel proteins are promiscuous and form assemblies with other proteins to produce a wide range of functional channel properties. In the *Drosophila* hearing organ, two members of the fly TRPV subfamily, Nanchung and Inactive, are required to form the channel pore⁸. One might expect something similar in vertebrate hair cells. There are indeed other TRP

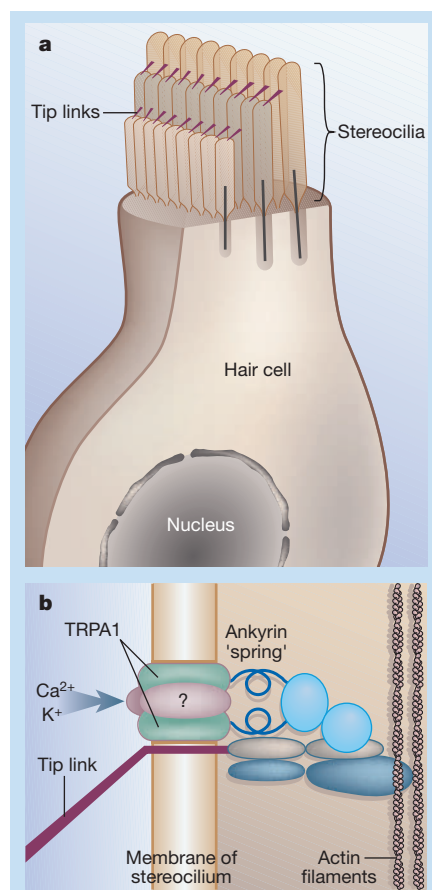


Figure 1 Converting sound and movement into electrical signals. **a**, The hair cell has an array of pencil-shaped stereocilia on its surface, each linked to its neighbour through a 'tip link'. **b**, The ion channel that mediates the conversion of sound or movement into electrical signals is located at one (and possibly both) ends of the tip link, which is shown here as a relatively stiff connection. The channel pore through which calcium (Ca^{2+}) and potassium (K^{+}) ions are transported is probably an assembly of four proteins², with TRPA1 as at least one of the subunits¹. The mechanism by which this channel is controlled is speculative, but it may involve a spring-like structural feature (with ankyrins forming the spring) and several other key proteins¹⁵ coupled into the actin core of the stereocilium.

candidates in hair cells, including TRPV1 (ref. 9) and TRPML3 (ref. 10), and these may form complexes with TRPA1.

In fact, the mix 'n' match habits of TRP channels might even be necessary to explain some cochlear biophysics. The kinetics of the hair-cell transducer varies along the cochlea, as the sounds being sensed change from low to high frequency^{11,12}, and it is possible that the gradation comes about as different TRPs form complexes. All the evidence suggests that stereocilia form extremely dynamic structures, with a high turnover of membrane and organellar constituents — just the sort of assembly ground for a transducer channel matched to hair-cell diversity.

The structure of the whole transducer complex still needs to be understood. How is this mechanosensitive channel controlled? An attractive hypothesis concerns a feature present in both NOMPC and TRPA1 — an iterating series of amino-acid motifs termed ankyrin repeats, found at one end (the amino-terminal end) of the proteins. There are 29 ankyrin repeats in NOMPC, and 17 in TRPA1. In NOMPC, the repeats form a whole turn of a helical spring at the cytoplasmic surface, which might couple displacement of the stereocilia to control of the channel¹³ (Fig. 1b). The structure of the vertebrate tip link suggests that most of the transducer compliance resides in cytoplasmic portions of the channel, not in the link¹, so the control spring is probably on the inside of the cell. The fewer ankyrin repeats in TRPA1 may indicate that it too has a helical spring at the cytoplasmic surface, but one that has only a partial turn. Such a spring would exert a torque when distorted, and it is not hard to see that this could rapidly control the channel when pulled or pushed by a tip link.

The highest-resolution electron micrographs show tip links splitting into two or three strands at the stereocilial tip¹⁴. Does this imply that several subunits of the channel are directly connected to the link, or are there further intermediates of the complex yet to be found¹⁵? The conundrum of which molecules associate together at the stereocilial tip remains. The presence of TRPA1, with its distinct structure, however, reopens the Pandora's box of the transduction field — the possibility that when the channel is opened, extracellular calcium entering through it directly alters the mechanics of the channel by acting on the helix structure. This local feedback might, if (and it's a big 'if') the phasing is right, even contribute to amplifying the very sounds that stimulate the hair cells in the first place. In any case, it is clear that we've not heard the last of TRPA1 and its associates. ■

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Obituary

Eberhard Gwinner (1938–2004)



Pioneer of circannual clock research

You don't have to be an ornithologist to be overawed by the spectacular annual migrations of many species of birds — anyone who has witnessed the tens of thousands of cranes or storks flying over the eastern Mediterranean in an astonishing show of synchrony and purpose will never forget the sight. But how is this migration triggered? How do birds navigate? How does migration link to other stages in the annual cycle of birds such as moulting and reproduction? Eberhard Gwinner's pioneering research over 40 years tackled many of these questions. Most notably, his work revealed a remarkable internal calendar, the circannual clock, which tells birds deep in the tropics that it is time to depart for their breeding grounds in Europe.

The concept of an internal daily rhythm (the circadian clock) was already established by 1964, when Gwinner completed his PhD on the social behaviour of ravens under the supervision of Gustav Kramer and Konrad Lorenz. Fascinated by biological rhythms, Gwinner moved to another department of the Max Planck Institute for Behavioural Physiology, situated in the beautiful Bavarian village of Andechs, famous for its beer and pink monastery. There he joined Jürgen Aschoff, one of the founding fathers of circadian clock research. Aschoff had shown that, when kept in constant environmental conditions, birds and other vertebrates, including humans, showed 'free-running' daily rhythms of behaviour and physiology; these rhythms, he concluded, were controlled by an internal clock. The fact that the rhythms deviated in predictable ways from 24 hours lent support to the notion that they were

not driven by some unidentified external cue but were indeed 'endogenous'.

It was initially thought that by measuring changes in day length, the daily clock could also act as a calendar for seasonal events. But Gwinner, struck by the fact that many animals live for part of the year in places where there is no obvious change in day length, yet time their annual events precisely, took a different view. Stimulated by Aschoff, he set out to test whether a separate endogenous clock might control annual, as opposed to daily, cycles. This was a daunting project, given that the equivalent of a ten-day experiment on circadian rhythms would take ten years for annual rhythms. As a staff scientist in the Max Planck Society, Gwinner was fortunate to have sufficient and secure long-term funding to embark on such a venture. This would be very difficult in the present-day university environment — one wonders what kinds of investigations have effectively become off limits because of their timescale.

For his project, Gwinner worked with captive migratory songbirds, notably willow and garden warblers, and stonechats. He showed that, even in constant photoperiodic conditions of 12 hours of light each day, the birds continued to show annual cycles of gonad growth, moult and migratory activity. These cycles persisted over many years and, just as free-running circadian rhythms differed from 24 hours, annual rhythms differed from 365 days. His longest-running experiments lasted for 12 years and are unlikely to be matched: they were a remarkable feat of vision, persistence, animal husbandry and rigour in record keeping. Following many twists and turns, Gwinner showed that

both the direction (measured in circular orientation cages) and duration of migration are controlled by internal annual time programmes, which are themselves influenced by seasonally changing environmental factors.

These circannual rhythms are thought to be linked to environmental changes via the circadian clock, but this matter remains unresolved. Some of Gwinner's most recent experiments, as yet unpublished, brought him closer to unravelling the mechanism he was seeking. But with his untimely death on 7 September 2004, answers to the question of whether there is a central generator of circannual rhythms, comparable to the suprachiasmatic nucleus, the master circadian clock in mammals, may now not emerge for a long time.

As a child, Gwinner was inspired by a local ornithologist, and instead of going into the family locksmith business he became a zoologist. He was an accomplished and enthusiastic naturalist, and made field trips to many parts of the world to collect birds for his research or to carry out fieldwork. Although much of his research involved analysing rhythms at the whole-organism level, he was interested in physiological mechanisms and embraced neurobiological and molecular techniques.

As a director of the institute in Andechs, he eschewed the normal trappings of his position: he was always more at home in shorts and open-necked shirt in the laboratory, involved in experiments or poring over data, than besuited in the boardroom. He ran his institute as a place where work and play merged into each other, and where all activities were motivated by questions and discussions about rhythm research and the latest results. In his last few years, he argued forcefully and successfully within the Max Planck Society for the creation of a Max Planck Institute for Ornithology, and thereby the continuation of ornithological research after his retirement.

'Ebo' Gwinner was very much a family man. His wife Helga, also a zoologist, worked with him, and he was immensely proud of his three children and five grandchildren. He was not only one of the most influential ornithological researchers of the second half of the twentieth century, but also a remarkable friend, colleague and mentor.

Roland Brandstaetter and John Krebs

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Nanotechnology

Nanoparticles go with the flow

Adv. Mater. doi:10.1002/adma.200401060 (2004)

In the absence of a solvent, nanoparticles behave like solids. But Athanasios B. Bourlinos *et al.* have come up with a way of changing nanoparticles into a liquid without using a solvent in which to dissolve or suspend them.

The authors coated silica nanoparticles (seven nanometres in diameter) with chemical groups borrowed from another burgeoning area of chemistry — ionic liquids. These chemical groups, made of long carbon chains with extra silicon and nitrogen atoms, give the nanoparticles a positive charge. By balancing this charge with a suitable negative ion, Bourlinos *et al.* created a 'nanosalt' whose physical properties could be tailored to make it a liquid at room temperature.

They performed the same trick using iron-oxide nanoparticles, producing a magnetic liquid that they describe as the first example of a solvent-free ferrofluid.

These liquid nanosalts, say Bourlinos *et al.*, are a unique class of solvent-free colloids, which could produce thin films of nanoparticles more easily or make useful solvents for chemical reactions. **Mark Peplow**

Genetics

Fragile fertility

Proc. R. Soc. Lond. B doi:10.1098/rspb.2004.2903 (2004)

When it comes to mating, it seems that males are the sensitive type. Ilik J. Saccheri and colleagues' study of butterfly fertility shows that the sterility effect resulting from inbreeding is largely restricted to males.

Inbreeding generally leads to a decrease in the genetic variability of a population, often with negative effects, because detrimental gene variants are not 'watered down'. Mated siblings of the *Bicyclus anynana* butterfly species, for instance, often produce eggs that do not hatch.

To investigate further, Saccheri *et al.* established six inbred lines of *B. anynana* and conducted various crosses, pairing males and females within and between these lines. By analysing the results of the different crosses, the authors deduced that inbred males were far more often to blame than inbred females for the reduction in egg-hatching. In other words, the reproductive fitness of males suffers more than that of females as a result of inbreeding.

Why this should happen remains uncertain. As the authors point out, the production of sperm has been suggested to be less developmentally stable than that of eggs, but this is probably not the only mechanism involved. **Roxanne Khamsi**



Fluid dynamics

Jove's jet engine

Geophys. Res. Lett. **31**, L22701 (2004)

Jupiter's colourful atmosphere has a characteristic banded pattern: 'zonal jets' flow in alternating directions, parallel to lines of latitude. Saturn's atmosphere has this structure too, and there are even faint zonal jets in Earth's oceans. Yet the origin of the banding is still not clear. The favoured hypothesis is that the jets are coherent structures that organize themselves within a shallow layer of turbulent fluid as a result of the planet's rotation thanks to the so-called 'planetary β effect' — the latitudinal variation of the Coriolis force.

Such an origin is supported by numerical calculations, but experiments mimicking a planetary atmosphere are challenging: to make the fluid sufficiently turbulent, the scale of the simulation needs to be very large. P. L. Read *et al.* have now achieved this by using a turntable 14 m in diameter, supporting a shallow layer of salty water in which convection (imitating that in the gas-giant atmospheres) is maintained by spraying denser, saltier water on top. The planetary β effect is reproduced topographically by a 'conical' slope in the bottom of the fluid tank. Small particles suspended in the flow reveal banded zoning, as small turbulent eddies conspire to generate a large-scale coherent pattern. The jets are not as neat as those on Jupiter, but they show that the basic idea works. **Philip Ball**

Biological hydration

Grease makes ions stickier

Phys. Rev. Lett. **93**, 228104 (2004)

Hydrophobic interaction — the apparent attraction between hydrophobic species in water — is considered a key factor in maintaining the correct folded conformation of a protein molecule. This attraction is thought to result, in a way that is still imperfectly understood, from changes in the arrangement of hydrogen bonds between water molecules surrounding a hydrophobe.

Florin Despa *et al.* have now identified a possible new role for water in protein folding. They calculate that water molecules in the hydration shell of a hydrophobic unit move an order of magnitude more slowly than those in the bulk, and that the dielectric constant of this water layer is significantly reduced.

Water's high dielectric constant is the reason it is a good solvent for ions: it screens their electrical charges, preventing them from aggregating. But in the vicinity of hydrophobic residues in a protein chain, the reduction in dielectric constant strengthens the attraction between charged residues, potentially helping to fix the protein's folds in place. Moreover, the effect means that water confined in nanoscale hydrophobic environments might have quite different solvent properties from those of the bulk liquid. **Philip Ball**

Microbiology

Stealth bacteria

Science doi:10.1126/science.1106036 (2004)

Michinaga Ogawa and colleagues have discovered how *Shigella*, a bacterium that causes diarrhoea, fever and stomach cramps, evades destruction by host cells. The feat is achieved by means of a camouflage protein that *Shigella* secretes.

The protein is called IcsB. Bacteria that lack it can still invade cells. But they persist for only about four hours, succumbing to a process in which the host cell wraps them up in the lamellar membrane of the autophagosome, an intracellular trap, where they are eventually destroyed.

How does IcsB enable the bacteria to evade capture for long enough to cause disease? The answer, say Ogawa and colleagues, lies in the bacterial surface protein VirG, a 'red flag' that is targeted by a host protein called Atg5, and which marks bacteria for trapping in the autophagosome. *In vitro*, Atg5 binds to VirG only when IcsB is absent. It seems that both Atg5 and IcsB can attach to the same binding region in VirG — implying that in host cells IcsB binds to VirG, concealing it from the host protein and allowing *Shigella* to go about its business undetected. **Michael Hopkin**

Creating a dry variety of quicksand

An aerated form of sand engulfs objects instantaneously, shooting out a jet of grains.

Sand can normally support a weight by relying on internal force chains^{1–3}. Here we weaken this force-chain structure in very fine sand by allowing air to flow through it: we find that the sand can then no longer support weight, even when the air is turned off and the bed has settled — a ball sinks into the sand to a depth of about five diameters. The final depth of the ball scales linearly with its mass and, above a threshold mass, a jet is formed that shoots sand violently into the air.

We allowed air to flow through very fine sand (typical grain diameter was about 40 μm), which was sitting in a container with a perforated base. The air stream was turned off before the start of the experiment and the sand allowed to settle (see supplementary information for details of methods). The packing fraction of this sand was only 41%, compared with 55–60% for untreated sand. We call this fragile state of sand ‘dry quicksand’ (not to be confused with normal quicksand, which is a mixture of sand, clay and water).

A ping-pong ball of radius $R=2.0$ cm, partly filled with bronze grains, was suspended above the treated sand so that it was just touching the surface. To release the ball without introducing any vibration, the thin rope supporting the ball was burned — causing the ball to sink instantaneously into the sand (Fig. 1). Objects often make a splash when they hit sand^{4,5}; in this case there was no splash, as expected, but a straight jet of sand shot violently into the air after about 100 ms^{4,5}. The singularity leading to this jet formation has already been described⁵.

In experiments using a ball that has a stiff, almost mass-less tail, which allows for tracking of the sinking depth as a function of time, we find that the ball reaches a final depth of $z_{\text{final}}=22.4$ cm (Fig. 2). This corresponds to more than five times its diameter. We investigated how this phenomenon is affected by the mass of the ball and found that the final depth reached by the ball increases linearly with its mass: that is, $z_{\text{final}} \propto m$. A visible jet only develops beyond a threshold mass of $m_{\text{thres}}=28.5$ g (Fig. 2).

We developed a simple force model to describe the dynamics of the ball in the sand and the parameter dependence of the final depth. The ball is accelerated by gravity and at the same time experiences a drag force, F_D , from the sand grains. For simplicity, we assume a Coulomb drag due to the normal forces from the side, which increases linearly with the depth z (ref. 6); pressure saturation due to sidewall support (Rayleigh–Janssen law) occurs only at much greater depth. We therefore have $F_D = -\kappa z$, where κ is a

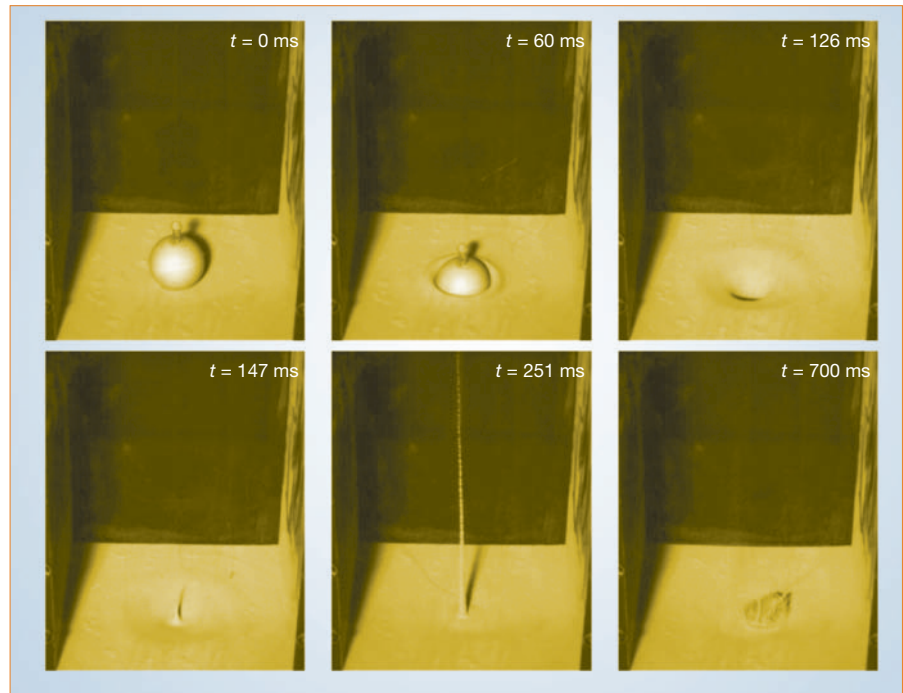


Figure 1 Snapshots of the sinking-ball experiment. At time $t=0$ ms, the ball (mass $m=133$ g) is released and immediately starts to sink into the sand; at $t \approx 130$ ms, a sand jet emerges, which reaches its final height at $t \approx 180$ ms. After about 600 ms, the trapped air bubble reaches the surface. (Experiment was built with assistance from G. W. Bruggert and input from C. Drösser.)

constant; note that F_D does not depend on velocity. The equation of motion is thus

$$(m + m_A)\ddot{z} = mg - \kappa z \quad (1)$$

where m_A is the ‘added mass’, meaning the mass of sand that is accelerated along with the moving ball. Equation (1) has to be supplemented by the boundary conditions $z(0)=0$ and $\dot{z}(0)=0$. Integration of equation (1) immediately gives the final depth,

$z_{\text{final}} = 2mg/\kappa$: that is, depth depends linearly on the mass. This agrees with the experimental findings shown in Fig. 2, from which we can read off $\kappa = 13.3 \pm 0.5 \text{ N m}^{-1}$. The full solution of equation (1) is

$$z(t) = \frac{1}{2} z_{\text{final}} (1 - \cos(\omega t)) \quad (2)$$

for $0 \leq t \leq \pi/\omega$, where $\omega = \sqrt{\kappa/(m + m_A)}$. Equation (2) describes the dynamics extremely well (Fig. 2, inset). From the fitted ω , we obtain m_A as zero, within measurement precision.

In nature, dry quicksands may evolve from the sedimentation of very fine sand after it has been blown into the air and, if large enough, might be a threat to humans. Indeed, reports that travellers and whole vehicles have been swallowed instantly^{7,8} may even turn out to be credible in the light of our results.

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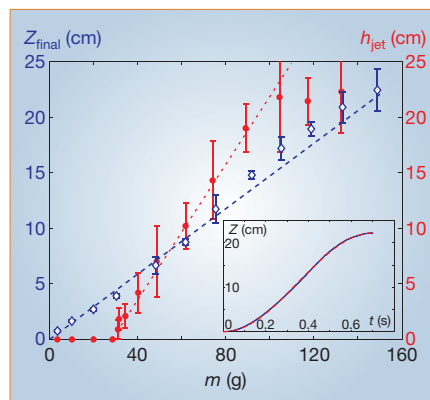


Figure 2 The jet height, h_{jet} (red circles), and the final depth of the ball, z_{final} (blue diamonds), as functions of the ball mass, m . Blue dashed line, linear fit with slope $2g/\kappa$; red dotted line, fit $h_{\text{jet}} \propto (m - m_{\text{thres}})^{1.0}$. The ball is released from rest from the surface of the sand (Fig. 1). Inset, depth of the ball, z , as function of time, t , for a ball of mass 148 g (experiment: blue curve; model: red curve).

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Adhesion

Elastocapillary coalescence in wet hair

We investigated why wet hair clumps into bundles by dunking a model brush of parallel elastic lamellae into a perfectly wetting liquid. As the brush is withdrawn, pairs of bundles aggregate successively, forming complex hierarchical patterns that depend on a balance between capillary forces and the elasticity of the lamellae. This capillary-driven self-assembly of flexible structures, which occurs in the tarsi of insects¹ and in biomimetic adhesives² but which can also damage micro-electromechanical structures^{3–6} or carbon nanotube ‘carpets’^{6–8}, represents a new type of coalescence process.

When the brush, which consists of regularly spaced, flexible lamellae, is progressively withdrawn from the bath of liquid, a cascade of successive sticking events leads to a hierarchical bundling pattern (Fig. 1a). We studied one such elementary sticking event for two lamellae separated by a distance d . If the strips were rigid, the perfectly wetting liquid would rise up to Jurin's height, $h_j = 2L_c^2/d$, where $L_c = (\gamma/\rho g)^{1/2}$ is the capillary length; γ and ρ are the liquid's surface tension and density, respectively. When the strips are flexible, capillary suction bends the lamellae and the liquid rises higher in this more confined environment. As two lamellae are withdrawn to height L (Fig. 1b), a capillary rise to height h_j (or to the top when $L < h_j$) precedes the sticking together of the strips, which happens when L becomes large.

Surprisingly, the height of rise L_{wet} increases linearly with L in this last regime, whereas $L_{\text{dry}} = L - L_{\text{wet}}$ remains constant. In fact, L_{dry} is prescribed by a balance between capillarity and elasticity. The capillary energy (per unit width) is $-2\gamma L_{\text{wet}}$, whereas the elastic energy is proportional to the square of the typical curvature, d/L_{dry}^2 , and reads exactly $3\kappa d^2/L_{\text{dry}}^3$ in this geometry, where κ is the bending stiffness of the strips. Minimizing the sum of the two energies (gravity becomes negligible in this regime) with respect to L_{dry} yields

$$L_{\text{dry}}^4 = \frac{9}{2} d^2 L_{\text{EC}}^2 \quad (1)$$

where $L_{\text{EC}} = (\kappa/g)^{1/2}$ is the elastocapillary length, in agreement with measurements made over several orders of magnitude

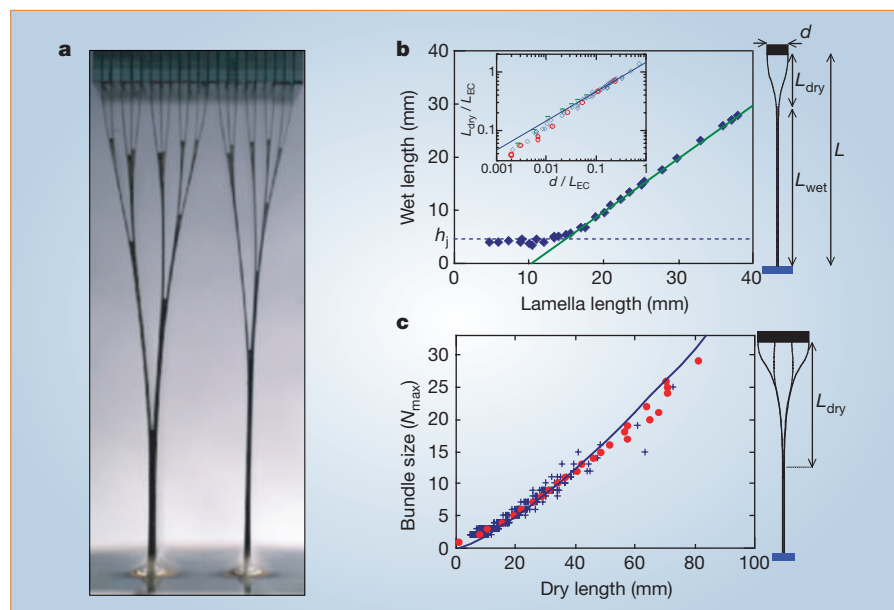


Figure 1 Flexible lamellae stick together after wetting. **a**, Lamellae in a wetted model brush after a sequence of sticking or unsticking events that cause aggregation (viewing from top to bottom) or fragmentation (from bottom to top), respectively. **b**, Height of rise, L_{wet} , of liquid between a lamella pair is plotted against the withdrawal height, L , showing the transition from the capillary rise (dashed line; $L_{\text{wet}} = h_j$) to the sticking regime (full green line; $L_{\text{dry}} = L - L_{\text{wet}}$ is constant). Polyester strips separated by $d = 1$ mm (width, 25 mm; thickness, $e = 100$ μm ; bending rigidity, $\kappa = 5.1 \times 10^{-4}$ N m) were dipped into silicon oil (density, $\rho = 950$ kg m^{-3} and surface tension $\gamma = 20.6$ mN m^{-1} , leading to $h_j = 4.3$ mm). Inset, sticking regime. Non-dimensional dry length, $L_{\text{dry}}/L_{\text{EC}}$, is plotted against non-dimensional separation, d/L_{EC} ; $L_{\text{EC}} = (\kappa/\gamma)^{1/2}$, which is the elastocapillary length (red circles: $e = 50$ μm , $L_{\text{EC}} = 47$ mm; blue diamonds: $e = 100$ μm , $L_{\text{EC}} = 150$ mm; green triangles: $e = 170$ μm , $L_{\text{EC}} = 370$ mm); line: comparison with theory (equation (1); no adjustable parameter). **c**, Aggregation of multiple lamellae into bundles ($e = 50$ μm , $d = 1$ mm). Number of lamellae per bundle is plotted against dry length. Blue crosses, raw data; red circles, averaging of data; line, comparison with theory (equation (2); no adjustable parameter).

(Fig. 1b, inset). An identical energy formulation is found in fracture theory⁹, in which the capillary energy is replaced by the material fracture energy. Whereas L_{EC} gives the typical curvature induced by capillarity¹⁰, L_{dry} is the critical length above which lamellar structures collapse. When the dimensions of a structure are scaled down by a factor λ , both L_{EC} and L_{dry} (scaling as $\lambda^{3/2}$ and $\lambda^{5/4}$, respectively) eventually become smaller than the structure size, an effect that is responsible for damaging microsystem structures^{2–8}.

To generalize equation (1) to multiple lamellae, we assume that a cluster of N lamellae behaves as a single lamella that is N times more rigid (we neglect solid friction as the wetting liquid lubricates the strips). On average, such a cluster results from the self-similar aggregation of two bundles of size $N/2$, clamped at a distance $Nd/2$. The dry length (above the junction of the two clumps) becomes

$$L_{\text{dry}}^4 = \frac{9}{16} N^3 d^2 L_{\text{EC}}^2 \quad (2)$$

which is in good agreement with experiment (Fig. 1c). The maximum size N_{max} of clusters in a brush with lamellae of length L is given by equation (2), with $L_{\text{dry}} = L$. However, smaller bundles are also seen if their aggregation with a neighbour leads to a size exceeding N_{max} . The broad distribution of clump sizes results from random initial imperfections, and requires statistical analysis. A

derivation based on Smoluchowski's coalescence process¹¹ leads to an original self-similar distribution that predicts an average cluster size of $0.67N_{\text{max}}$ (A. B. *et al.*, manuscript in preparation).

Our results, once scaled down, could help to improve the design of micro-electromechanical systems. The self-similar aggregation process described here should extend to different geometries (such as those of fibrous materials) and to similar systems involving coalescence or fragmentation.

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Bose–Einstein condensation of excitons in bilayer electron systems

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An exciton is the particle-like entity that forms when an electron is bound to a positively charged ‘hole’. An ordered electronic state in which excitons condense into a single quantum state was proposed as a theoretical possibility many years ago. We review recent studies of semiconductor bilayer systems that provide clear evidence for this phenomenon and explain why exciton condensation in the quantum Hall regime, where these experiments were performed, is as likely to occur in electron–electron bilayers as in electron–hole bilayers. In current quantum Hall excitonic condensates, disorder induces mobile vortices that flow in response to a supercurrent and limit the extremely large bilayer counterflow conductivity.

In many-particle quantum physics bosons are special. Unlike the ubiquitous electrons and their fermionic cousins, any number of bosons can crowd into the same microscopic state. Indeed, Einstein predicted in 1924 that at low temperatures essentially all of the bosons in a macroscopic system would spontaneously ‘condense’ into the same low-energy quantum state.

In quantum mechanics, an individual particle is represented by a wave that has both amplitude and phase. The most remarkable consequence of Bose–Einstein condensation (BEC) of a vast number of particles is that macroscopic properties become dependent on a single wavefunction, promoting quantum physics to classical time- and length scales. A BEC is a highly ordered state in which the wavefunction phase is coherent over distances much longer than the separation between individual particles. Long-range quantum phase coherence has many dramatic physical consequences, of which the most spectacular is superfluidity, that is, the ability of matter to flow around obstacles with extremely weak, often immeasurable, dissipation.

Buckets of bosons

All known BECs are formed by particles that are actually composite bosons made up of an even number of fermions. Helium-4 atoms, for example, contain two protons, two neutrons and two electrons, and Bose condense to form a superfluid at just a few degrees above absolute zero. Today there is intense interest in BECs in rarefied atomic vapours. In superconductors electrons form pairs at low temperatures. These ‘Cooper pairs’ are again composite bosons. In this case, the formation of pairs and their collective Bose condensation usually occur at the same low temperature; the transition temperature is controlled by the underlying fermionic physics, not purely by bosonic quantum mechanics. Correspondingly, the average separation between electrons in a given Cooper pair usually greatly exceeds the mean distance between pairs.

An exciton in a semiconductor consists of an electron bound to a hole. In most cases the electron is in the conduction band of the semiconductor, and the hole, which is nothing more than an unfilled electronic state masquerading as a positively charged object, is in the valence band. Excitons are usually created by shining light on the semiconductor, which creates electrons and holes in equal numbers. Optically generated excitons like these are ephemeral objects, decaying quickly via the emission of light.

Electrons and holes are both fermions, but excitons are bosons. After the 1957 pairing theory of superconductivity was solidly

established, physicists began to speculate¹ about BEC of excitons in semiconductors. The short lifetime of optically generated excitons has been a major obstacle to realizing this phenomenon^{2,3}. Indirect excitons in bilayer quantum well systems have two key advantages over bulk systems and have thus played an increasingly important role in this field^{4,5}. Quantum wells are realized in layered semiconductor structures and allow for the confinement of electrons and holes to two-dimensional (2D) planes. Indirect excitons are bound states of conduction-band electrons in one well and valence-band holes in an adjacent well. The separation between electrons and holes reduces the rate at which they recombine into photons. In addition, the spatial separation between electrons and holes causes the excitons to act like oriented electric dipoles that have repulsive interactions. The repulsion is important because it prevents the electrons and holes from agglomerating into an uninteresting electron–hole plasma. Experimental studies of optically generated indirect excitons have uncovered a number of fascinating many-body effects^{6,7}. The enhanced exciton mobility, increased radiative decay rate, and photoluminescence noise that has been reported in these systems at low temperatures and high fields, all suggest collective, possibly coherent, behaviour. Still, the requirements of high density and low temperatures are difficult to achieve in this non-equilibrium system, and there is not yet compelling evidence for an exciton BEC.

Exciton condensation is closely analogous to other common electronic orders in which electron–hole pairs condense. For example, charge-density-wave states are formed by condensation of electron–hole pairs located at different points in a crystal’s Brillouin zone and ferromagnetism can be regarded as being due to condensation of pairs formed from electrons and holes with different spin indices. The analogies are never complete, however—the broken translational symmetry in charge-density-wave states introduces a number of qualitative distinctions, for example. Ferromagnetism in systems with nearly uniaxial easy-plane anisotropy is perhaps most closely analogous to exciton condensation. Indeed, it has been argued that some of the phenomena that have been studied in the bilayer electron systems of interest here, including collective condensate transport, can also occur⁸ in thin-film metallic ferromagnets.

Excitons in electron–electron bilayers

Here we describe compelling evidence that exciton condensation has recently been discovered, not in electron–hole systems as expected, but in systems with two parallel layers of conduction-band electrons

like those illustrated schematically in Fig. 1. Importantly, the excitons in this system are present in equilibrium, waiting patiently for experimenters to reveal their properties.

The techniques required to grow high-quality single and bilayer electron systems are now well established and have been vital in a great many important physics discoveries, notably the famous fractional quantum Hall effect⁹. At first sight it seems impossible to achieve BEC of excitons in electron–electron bilayers, because all the fermions have repulsive interactions. Quantum well electrons are, however, able to perform remarkable tricks when placed in a strong perpendicular magnetic field.

The Lorentz force bends classical electron trajectories into circles—cyclotron orbits. In a 2D quantum system the kinetic energy of these orbits is quantized into discrete units. The set of orbits with a particular energy, a ‘Landau level’, is, however, highly degenerate because the tiny cyclotron orbits can be positioned all across the 2D plane. It turns out that the number of degenerate states in the lowest-energy Landau level is equal to the number of quanta of magnetic flux that pass through the electron layer. At high magnetic field it is easy to enter a regime in which the total number of electrons is less than the number of states in the lowest Landau level. In Fig. 1, for example, we illustrate the circumstance in which the number of electrons in each layer is one-third of the number of available states; this is referred to as filling factor $\nu = 1/3$.

BEC at strong magnetic fields in electron–electron bilayers is most easily understood by making a particle–hole transformation in one of the two layers; we have chosen the bottom layer for this in Fig. 1. The particle–hole transformation^{10,11} is one in which we simply keep track of the empty states in the Landau level rather than the full ones. It is mathematically exact, changes the filling factor of the transformed layer from ν to $1 - \nu$, and changes the sign of the carrier charge from negative to positive. The interaction of holes with electrons in the untransformed layer is thus attractive.

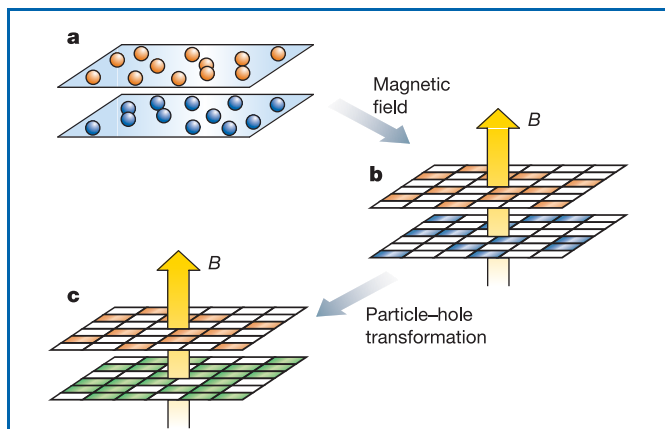


Figure 1 An electron–electron bilayer system in a strong magnetic field is equivalent to an electron–hole bilayer. **a**, Cartoon depiction of two parallel layers of electrons. **b**, In a magnetic field the kinetic energy of 2D electrons is quantized into discrete Landau energy levels. Each such Landau level contains a huge number of degenerate orbitals, here depicted schematically as a checkerboard of sites. If the field is strong enough, all electrons reside in the lowest Landau level, and only occupy a fraction (here one-third) of the available sites. **c**, A particle–hole transformation applied to the lower electron layer places the emphasis on the unoccupied sites—that is, the holes (coloured green) in that layer. This transformation, which is formally exact and completely equivalent to the more familiar transformation used to describe empty valence-band states in a semiconductor as holes, changes the sign of the Coulomb interactions between layers from repulsive to attractive. Exciton BEC occurs when holes in the lower layer bind to electrons in the upper layer. This is most likely to occur when the number of electrons and holes are equal, that is, when each layer is half-filled (this is not the case in this figure).

Particle–hole transformations also change the sign of the kinetic energy, from negative to positive for valence-band holes at zero magnetic field for example. Because the kinetic energy is the same for all electrons and holes in the lowest Landau level, BEC at strong fields is just as likely to occur in electron–electron bilayers as in electron–hole bilayers.

This last point is a subtle one; indeed, spontaneous coherence¹² and superfluidity¹³ were predicted in electron–electron bilayers without explicitly recognizing their equivalence to earlier predictions¹⁴ for electron–hole bilayers. We emphasize, however, that particle–hole transformation of a conduction-band Landau level is completely equivalent to the much more familiar transformation used to map unoccupied valence-band electron states into holes.

In Fig. 1 the number of holes in the bottom layer exceeds the number of electrons in the top layer. To create an exciton BEC, these populations should be nearly equal; one should therefore begin with half-filled Landau levels in each layer. Early experiments proved that in this $1/2 + 1/2$ situation a bilayer electron system can exhibit a quantized Hall effect¹⁵. In other words, its Hall resistance, measured with electrical currents flowing in parallel through the two layers, is precisely equal to h/e^2 , Planck’s constant divided by the square of the electron charge. This remarkable effect results from a complex interplay of Landau quantization, Coulomb interaction effects, and imperfections in the 2D plane. Although observation of a quantized Hall effect in the bilayer system at this filling factor demonstrates the importance of interlayer Coulomb interactions, it does not on its own suggest the existence of an exciton BEC. The hunt for BEC requires different experimental tools.

Experimental evidence for exciton formation

In a bilayer 2D electron system the two quantum wells are separated by a thin barrier layer. By adjusting the thickness and composition of this barrier, it is possible for electrons in one layer to interact strongly, via the Coulomb interaction, with the electrons in the

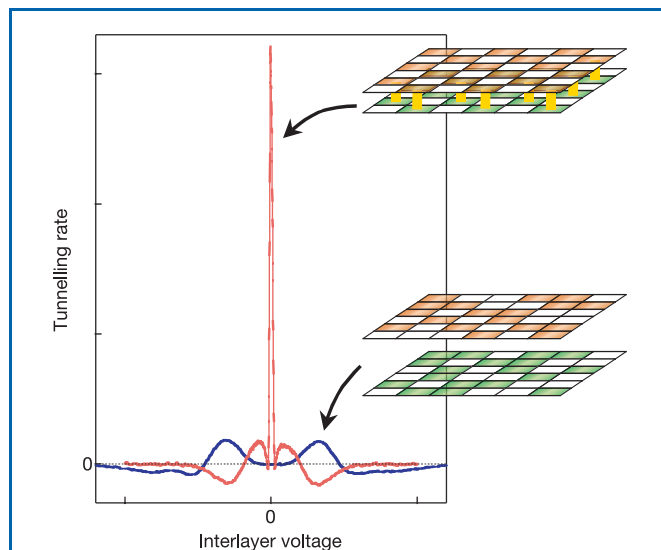


Figure 2 Tunnelling rate versus interlayer voltage in a bilayer electron system. These traces are actual data, and are taken at magnetic fields where exciton condensation is most expected (that is, one half-filling of the lowest Landau level per layer). In the blue trace the layers are relatively far apart, whereas in the red trace they are closer together. The dramatic difference between them is a direct indication that a phase transition in the bilayer system occurs when the layer separation is reduced below a critical value. The huge enhancement of the tunnelling rate at zero energy in the red trace points to interlayer electron–hole correlations: that is, it suggests that every electron is positioned opposite a hole into which it can easily tunnel.

other layer, and yet have an extremely small probability of quantum mechanically tunnelling through the barrier. Achieving this strong-correlation, weak-tunnelling limit is key to the occurrence of excitonic Bose condensation in the bilayer 2D electron system, as we discuss further below.

Figure 2 shows measurements¹⁶ of the rate at which electrons tunnel between the two layers of a bilayer 2D electron system as a function of voltage difference. These data were taken at very low temperatures and with the magnetic field adjusted to produce the half-filling per layer condition. The two traces in the figure differ only in the effective interlayer separation. For the blue trace, the layers are relatively far apart. For voltages near zero, the tunnelling rate is very small. This suppression of the tunnelling is not sensitive to small changes in the magnetic field and grows more severe as the temperature is reduced. Often referred to as a Coulomb gap, the effect is a consequence of strong correlations between electrons—within the individual layers. Tunnelling at low voltages (or, equivalently, low energies) is suppressed because an electron attempting to enter a 2D electron layer at high magnetic field blunders disruptively into the delicate dance being performed by the electrons in that layer as they skilfully avoid one another. This clumsy entry can only produce a highly excited, non-equilibrium state and can occur only at high voltage. Although quite interesting in its own right, the effect obviously does not suggest exciton condensation.

The red trace in Fig. 2 is dramatically different. A slight reduction in the effective layer separation has produced a giant peak in the tunnelling rate. In sharp contrast to the suppression effect in the blue trace, this peak grows rapidly in height as the temperature is reduced and is very sensitive to magnetic field. Small changes in the field away from the value needed to produce a half-filled lowest Landau level rapidly destroy the peak.

The stark difference between the two traces in Fig. 2 suggests that a quantum phase transition occurs as a function of effective layer

separation. The strong peak in the tunnelling rate seen at small separations is inconsistent with the arguments used to understand the suppression effect at larger separations. Instead of being unaware of the correlations in the layer about to be entered, the tunnelling electron is apparently already participating in the dance. Indeed, the peak suggests that all electrons are strongly correlated with their neighbours in both layers. Moreover, the temperature and magnetic-field dependence of the peak point to the existence of a new and intrinsically bilayer collective state in which electrons in one layer are always positioned opposite holes in the other layer.

Strong electron–hole correlations are necessary but not sufficient for exciton condensation. To make the argument for excitonic BEC in a bilayer 2D electron system more compelling, an experiment demonstrating the transport of electron–hole pairs is needed. But how does one move and detect neutral objects? The key is to note that the uniform flow of excitons is equivalent to ordinary electrical currents flowing in opposite directions in the two layers. Technical tricks have allowed us to make independent electrical connections to the individual layers in bilayer electron samples¹⁷. With these contacts it is easy to arrange for equal and opposite currents to flow in the two layers and directly test whether or not excitons are available to perform the particle transport.

Figure 3 shows a cartoon version of what is expected from such a counterflow measurement. The two traces represent the Hall voltages expected in the two layers, neglecting all quantum effects except exciton condensation. Because of the Lorentz force on a moving charge, the Hall voltage is, in most cases, simply proportional to the magnetic field. In a bilayer system with counterflowing currents, the Hall voltages in the two layers will be of opposite sign.

If the distance between the two layers is too large, or if the magnetic field is far from the half-filling per layer condition, exciton condensation will not occur. If, however, the layers are closely spaced and the magnetic field is just right, interlayer electron–hole pairs will form and carry the counterflow current. The Hall voltage in each layer should then drop to zero, as suggested in the figure. This prediction can be understood in a variety of ways, most simply by observing that excitons are neutral and thus feel no Lorentz force. Without the Lorentz force the Hall voltage must vanish. This remarkable prediction has recently been confirmed by our own group at Caltech¹⁸ and the effect has been reproduced, in a slightly different bilayer system, by researchers at Princeton¹⁹. The same experiments do, however, show that the electron–hole transport current flows with a weak but measurable dissipation. The activated temperature dependence of the dissipation is inconsistent with independent exciton transport, because the exciton diffusion constant should then approach a constant as the temperature goes

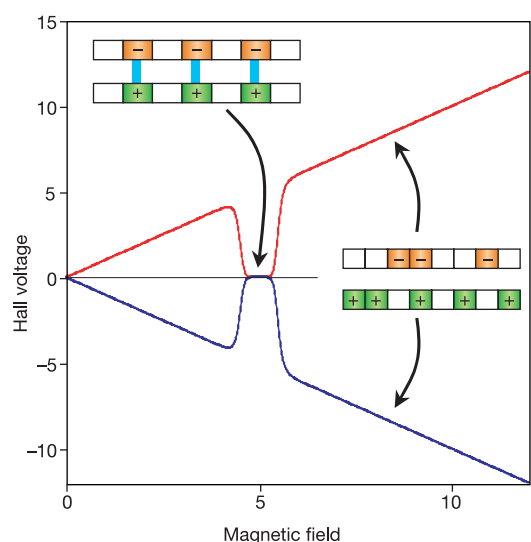


Figure 3 Hall voltage measurements reveal exciton condensation. The two traces schematically indicate the Hall voltages in the two electron layers when the electrical currents flowing in them are oppositely directed. All quantum effects, except exciton condensation, have been ignored. When the currents are carried by independent charged particles in the two layers, non-zero Hall voltages must be present to counteract the Lorentz forces. Because the currents are oppositely directed, these voltages have opposite signs in the two layers. However, if exciton condensation occurs at some magnetic field, the oppositely directed currents in the two layers are carried by a uniform flow of excitons in one direction. Being charge-neutral, these excitons experience no Lorentz force and the Hall voltage is expected to vanish. This remarkable effect has very recently been definitively observed.

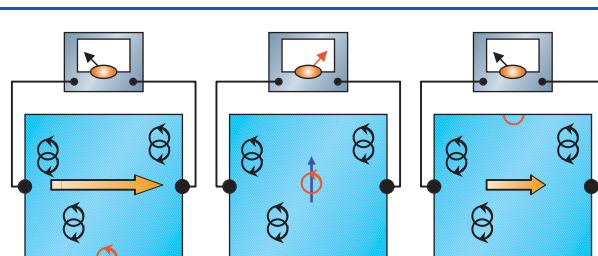


Figure 4 Motion of unpaired vortices leads to dissipation in excitonic superfluids. The elementary excitations in quantum Hall excitonic superfluids are vortices that carry electrical charge and topological charge. In an ideal system at low temperatures vortices occur in bound pairs and do not contribute to the decay of excitonic supercurrents. Disorder, however, is present in all real samples and is capable of producing unpaired vortices. The weak dissipation observed in recent counterflow experiments is associated with the activated transport of such unpaired vortices.

to zero. Instead, these new results can only be explained in the context of collective exciton-condensate transport.

Excitonic superfluidity

Quantum mechanics is invariant under a global change in wavefunction phase, and the spatial phase variation rate determines velocity. In an excitonic BEC the same simple description holds. The velocity of a gas of electron-hole pairs is proportional to the spatial gradient of the condensate phase, and excitonic superfluidity requires the absence of microscopic processes that favour a particular value of the condensate phase. The presence of these processes would invalidate the analogy between the quantum mechanics of the condensate and that of a simple quantum particle. Because any process that transfers electrons between the two bands that form the exciton will be sensitive to the condensate phase, and such processes are not forbidden by symmetry, ideal excitonic superfluidity is—strictly speaking—an impossibility²⁰. The dominant condensate-phase-sensitive process in bilayers is interlayer tunnelling, which either creates or destroys an exciton. It is ironic that it was the measurements¹⁶ of this weak process, discussed above, that provided the first compelling evidence for exciton condensation. Experimentally, these processes are expected^{21,22} to lead to nonlinearities in the counterflow current-voltage relationship. In our opinion, a full description of the influence of interlayer tunnelling on counterflow transport raises theoretical questions that are fundamentally new and not well understood. The absence of nonlinearities in current experiments suggests that this physics has been masked by dissipation due to the motion of vortices that are nucleated in great numbers in these quantum Hall samples by disorder. We expect that the influence of tunnelling on counterflow transport will become apparent in samples with stronger interlayer tunnelling amplitudes than those studied until now, and in current samples at sufficiently low temperatures.

Because the condensate wavefunction is collective, elementary quantum processes which act on one particle at time cannot effectively reduce the velocity of a BEC. In 2D superfluids, condensate current decay is limited instead by the nucleation and transport of vortices: points around which the condensate phase changes by 2π . The elementary process which leads to phase gradient decay is one in which a vortex moves across the system, as illustrated in Fig. 4. The energy required to create an isolated vortex in the ground state of an ideal 2D BEC is (logarithmically) infinite. At temperatures below the Kosterlitz-Thouless transition temperature, at which the free energy of an isolated vortex vanishes, no unpaired vortices should be present.

Vortices are especially important in bilayer exciton condensates because they tend to be nucleated by disorder. The special role of vortices in these superfluids can be understood by thinking about the Aharonov-Bohm phases associated with the external magnetic field. As explained above, the number of states N in a Landau level is proportional to the number of quanta of magnetic flux that penetrate the 2D layers. Correspondingly, the total phase change of an electron orbital that encircles the system is $2\pi N$. A vortex in the condensate phase therefore either increases or decreases the number of states that can be accommodated in one of the layers by a single unit. Vortices consequently come in four flavours²³ and carry charge equal in magnitude to one-half of the electron charge. In bilayer exciton condensates vortices couple to, and are therefore nucleated by, external disorder potentials. The experimental results discussed above demonstrate that vortex motion in the present excitonic BECs requires only a finite activation energy. In all current samples, free vortices are present down to the lowest temperatures studied.

The road from the early suggestions that there might be an excitonic analogue of superconductivity to these recent discoveries has been marked by unanticipated twists and turns. Excitonic BEC

requires strong inter-band interactions, but a very small amplitude for transitions between bands that are due to either one-body potentials or interactions. The possibility of studying the bilayer quantum well systems that satisfy this requirement so beautifully had to wait for the development of the techniques we now have for growing extremely high quality layered semiconductors. The interaction energy gained by excitonic condensation must compete with band energies and energy gains associated with other competing types of order. The understanding that Landau quantization in a strong field could eliminate the band energy cost (even in electron-electron bilayers), and that excitonic condensation could prevail over other orders under some circumstances, grew out of the theory of the fractional quantum Hall effect—something that had not been anticipated at the beginning of this journey. Finally, the question of how collective transport of neutral objects could be detected in an excitonic BEC has always been a thorny issue. The separate contacting techniques that enable counterflow transport measurements, developed with quite different goals in mind, provide a direct solution to this problem. In science as in life, way leads onto way, and destinations are often reached by unanticipated paths. □

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Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution

International Chicken Genome Sequencing Consortium*

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We present here a draft genome sequence of the red jungle fowl, *Gallus gallus*. Because the chicken is a modern descendant of the dinosaurs and the first non-mammalian amniote to have its genome sequenced, the draft sequence of its genome—composed of approximately one billion base pairs of sequence and an estimated 20,000–23,000 genes—provides a new perspective on vertebrate genome evolution, while also improving the annotation of mammalian genomes. For example, the evolutionary distance between chicken and human provides high specificity in detecting functional elements, both non-coding and coding. Notably, many conserved non-coding sequences are far from genes and cannot be assigned to defined functional classes. In coding regions the evolutionary dynamics of protein domains and orthologous groups illustrate processes that distinguish the lineages leading to birds and mammals. The distinctive properties of avian microchromosomes, together with the inferred patterns of conserved synteny, provide additional insights into vertebrate chromosome architecture.

Genome sequence comparison is a modern extension of the long-standing use of other species as models to illuminate aspects of human biology and medicine. Large-scale genome analyses also highlight the evolutionary dynamics of selective and mutational processes at different chronological scales^{1–4}. We present here results obtained from an extensive analysis of a draft sequence of the chicken genome, which has evolved separately from mammalian genomes for ~310 million years (Myr)^{4,5} (Fig. 1). This genome is the first to be sequenced at this particular evolutionary distance from humans, and, as shown previously^{6–8}, it provides an excellent signal-to-noise ratio for the detection of functional elements. Our analysis of the $6.6 \times$ coverage draft sequence of the chicken genome resulted in the following main observations.

- The nearly threefold difference in size between the chicken and mammalian genomes reflects a substantial reduction in interspersed repeat content, pseudogenes and segmental duplications within the chicken genome.
- Chicken–human aligned segments tend to occur in long blocks of conserved synteny. We find a relatively low rate of chromosome translocations in both lineages from the last common ancestor, whereas intrachromosomal rearrangements (for example, inversions) are more common.
- Syntenic relationships for certain classes of non-coding RNA (ncRNA) genes differ from those of protein-coding genes. This observation implies a novel mode of evolution for some ncRNA genes.
- Expansion and contraction of multigene families seem to have been major factors in the independent evolution of mammals and birds.
- The sizes of chicken chromosomes, which span a range of nearly two orders of magnitude, correlate negatively with recombination rate, G+C and CpG content, and gene density but positively with repeat density.
- Synonymous substitution rates are elevated for genes in both chicken microchromosomes and in subtelomeric regions of macrochromosomes.
- There is a paucity of retroposed pseudogenes in the chicken genome, in contrast to mammalian genomes, greatly simplifying the classification of chicken gene content. This is explained by the

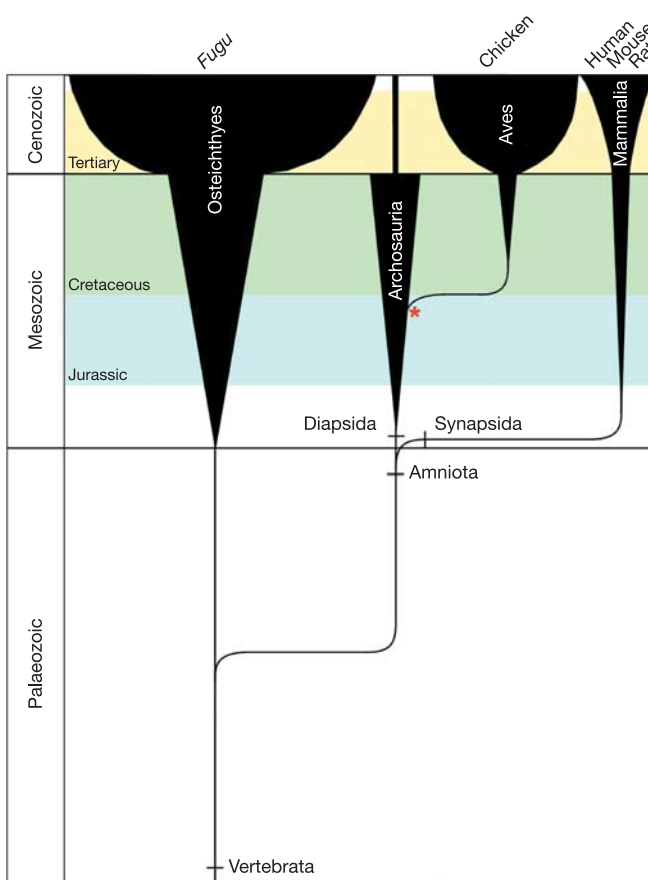


Figure 1 Basal vertebrate evolution showing extant species whose genomes have been sequenced. The horizontal axis represents estimated relative species diversity. The Archosauria include the Aves, their Mesozoic dinosaur predecessors, and Crocodilia; the Lepidosauria (lizards, snakes and tuataras) are not indicated. Archaeopteryx (indicated by an asterisk) is considered to be the first known bird and lived approximately 150 Myr ago. See also ref. 159.

high specificity of the reverse transcriptase from the predominant interspersed repeat element in the chicken genome: the CR1 long interspersed nucleotide element (LINE).

- Unlike all other vertebrate genomes studied so far, no short interspersed nucleotide elements (SINEs) have been active in the chicken genome for the last ~50 Myr.
- Alignment of the chicken and human genomes identifies at least 70 megabases (Mb) of sequence that is highly likely to be functional in both species.
- Many of the chicken–human aligned, non-coding sequences occur far from genes, frequently in clusters that seem to be under selection for functions that are not yet understood.

Perspectives on the domestic chicken

The chicken (*Gallus gallus*) is an important model organism that bridges the evolutionary gap between mammals and other vertebrates and serves as the main laboratory model for the ~9,600 extant avian species. The chicken also represents the first agricultural animal to have its genome sequenced. Modern birds (Ornithurae) evolved from theropod dinosaurs^{9,10} in the middle of the Mesozoic era (Fig. 1). Chickens were domesticated in Asia at least by 5400 BC, perhaps as early as 8000 BC^{11–13}. Darwin¹⁴ suggested that the red jungle fowl was the nearest ancestor to the domestic chicken, a view later confirmed by mitochondrial DNA analysis¹⁵.

Genetic analysis of the chicken dates back to the start of the twentieth century^{16,17}, and hundreds of well-characterized mutant stocks and inbred lines have been developed¹⁸. The chicken embryo has been an especially useful vertebrate system for developmental biologists¹⁹ owing to experimental advantages of *in ovo* embryogenesis. Furthermore, the chicken has been used in seminal studies in virology, oncogenesis and immunology^{20–22}. The chicken genetic linkage map, initiated early in the last century²³, now includes 2,172 genetic loci with a total length near 4,000 cM^{24,25}. Most avian karyotypes contain chromosomes of markedly different lengths, termed the macro- and microchromosomes, and thus bird karyotypes are quite distinctive as compared with those of mammals²⁶. The chicken karyotype ($2n = 78$) is made up of 38 autosomes and one pair of sex chromosomes, with the female as the heterogametic sex (ZW female, ZZ male).

Sequencing and assembly

All sequencing libraries were prepared from DNA of a single female of the inbred line of red jungle fowl (UCD 001) to minimize heterozygosity and provide sequence for both the Z and W sex chromosomes, albeit at 50% of the autosomal coverage. The assembly was generated from ~6.6 × coverage in whole-genome shotgun reads, a combination of plasmid, fosmid and bacterial artificial chromosome (BAC)-end read pairs (Supplementary Table S1). The assembly (Table 1) was generated using PCAP²⁷, a parallel algorithm that exploits both read-pairing constraint information and base quality values (see Supplementary Information for a description of the methods).

A BAC-based physical map for the chicken was developed in parallel with the sequence assembly²⁸. Along with the genetic map^{25,29–31}, this provides the main scaffolding for the assembly into larger ordered and oriented groupings ('ultracontigs') as well

as the mechanism for chromosomal assignment (see Methods). After integrating data from the physical map with the assembly, several additional steps were taken to improve the initial assembly of chicken chromosome sequences. This included using expressed sequence tag (EST) and messenger RNA data to aid the ordering and orientation of sequence, and using map and sequence data to aid in localization of centromeres and telomeres (see Methods). The resulting assembly consists of 574 segments made up of 84 ultracontigs (ordered and oriented by their relationship to the physical map) and 490 'supercontigs' (ordered and oriented by read-pairing data, but not linked to the physical map) anchored to chicken chromosomes. Of the 1.05 gigabases (Gb) of assembled sequence, 933 Mb were localized to specific chromosomes, 907 Mb of which were ordered and oriented along those chromosomes.

Assessment of the coverage and quality of the genome assembly

We estimated the coverage of the assembly using both finished BACs and available mRNA sequences (Methods). In a set of 38 finished autosomal BAC sequences from the same red jungle fowl female (covering 6 Mb of sequence), 98% of finished bases could be aligned with the draft whole-genome shotgun assembly, with an overall substitution rate of 0.02% and no deletions or insertions. Similarly, of a set of 23,212 chicken mRNAs and 485,000 ESTs³², 97% and 96% respectively are at least partially found in the assembly. Of these, 10% are only partially found or are fragmentary. This lack of contiguity contributes in part to the 5–10% of genes estimated to be absent from the Ensembl chicken gene set (see below). Representation of the (G+C)-rich extremes of the genome may be less complete. In one small region of incompletely sequenced BACs (3.6 Mb) orthologous to human chromosome 19 (HSA19) (I. Ovcharenko *et al.*, unpublished data), where the average G+C content was 52% (with some regions exceeding 60%), coverage fell to 82%. Furthermore, we examined a set of 400 genes that were represented in chicken mRNA or ESTs and had single orthologues in five diverse species (human, mouse, rat, *Takifugu rubripes* (*Fugu*) and *Drosophila*) but were predicted to be absent from, or at least partially truncated in, the chicken Ensembl gene set (see below). Over 70% were in fact partially found in the assembly. Of the 400, the largest fraction missing (21%) were HSA19 orthologues from a region known to be unusually rare in chicken clone libraries (L. Gordon *et al.*, unpublished data). The missing genes have a higher G+C content than average and many, including some HSA19 orthologues, are associated with intronic simple sequence repeats (see Methods).

Comparisons to 6 Mb of finished red jungle fowl BAC clone sequence revealed alignment with 311 chicken contigs from 62 supercontigs, which were used to assess possible ordering errors (see Methods). No orientation problems were detected, and only two order discordances (misordered sequence contigs within a supercontig) were discovered. This would extrapolate to a total of ~400 kilobases (kb) of misordered contigs in the current assembly. In addition, eight cases were found in which a contig was incorrectly inserted into a supercontig, equivalent to ~1 Mb of incorrect insertions in the full genome.

Recent duplications are especially difficult to place within whole genome assemblies. Relaxed assembly may collapse duplicated segments into one, and stringent assembly may break sequences into duplicates because of sequencing errors. In the chicken, 'all-versus-all' comparison shows that 11% (~123 Mb) of the genome sequence is in pairwise alignments larger than 1 kb with more than 90% sequence identity. The bulk (91% of the 11%), however, are highly similar (>98%) and might represent false duplication. In a direct test (see Methods), only 22% of duplications with near-perfect sequence identity (>98%) and 26% (32.3 out of 122.7 Mb) of the full set were confirmed.

Because the assembly process incorporated genetic markers (Supplementary Table S2), the genetic map does not provide

Table 1 Whole-genome assembly statistics

Genome feature	>1 kb number	N50 length (kb)	N50 number	Largest (kb)
Contigs	98,612	36	7,486	442
Supercontigs	32,767	7,067	37	33,505

Statistics presented are for the whole-genome assembly before integration of physical mapping data. Contigs are contiguous sequences not interrupted by gaps, and supercontigs are ordered and oriented contigs including estimated gap sizes. The N50 statistic is defined as the largest length *L* such that 50% of all nucleotides are contained in contigs of size at least *L*. A total of 10,743,700 reads were included in the final assembly. Only 4.39% of the total sequencing reads presented to the assembler were not used in the final assembly.

independent assessment. However, recent placement of 142 additional genetic markers, mapped after the assembly, suggests that less than 0.75% (~6 Mb) of the sequence has been assigned to a wrong chromosome. Thus, the assembly correctly places the vast majority of the chicken genome in long contiguous stretches. It is well ordered and oriented and faithfully represents older segmental duplications (at the cost of a modest false increase in the most recent duplications). The draft provides an excellent substrate for initial global analysis, recognizing that the elucidation of the full sequence will be critical to allow final, definitive conclusions.

Gene content of the chicken genome

The genome sequence of an organism encodes both ncRNAs and proteins. Extensive analysis of the genome sequences of human¹, mouse² and rat³ has provided our current best assessment of mammalian gene content and has illuminated much about the evolution of genes. The chicken genome provides new perspectives on both the structure and content of mammalian genes, as well as yielding insight into avian gene content and evolution of ncRNA genes.

Non-coding RNA genes

A total of 571 ncRNA genes, from over 20 distinct gene families, were identified within the chicken genome assembly (Table 2) using bioinformatic approaches^{33,34} (see Methods). Predicted ncRNA pseudogenes are greatly reduced in number relative to their human ncRNA counterparts. The chicken ncRNA predictions therefore represent a set that is mainly functional. If ncRNA genes maintain their placement with respect to neighbouring genes, chicken ncRNA gene locations could be used to identify which mammalian copies are likely to be functional and which are probable pseudogenes. However, few chicken and human ncRNA genes are paired in regions of conserved synteny (Table 2), relative to the high level of shared gene order observed for protein-coding genes (see below). Those classes of ncRNAs that are most often syntenic are microRNAs (miRNAs) and small nucleolar RNAs (snoRNAs), which are often found in the introns of protein-coding genes (or, rarely, of specialized 'host' genes³⁵). Most ncRNA genes

thus seem to have been translocated to distant genomic sites during vertebrate evolution, without accumulating large numbers of pseudogenes, as would be expected were this process to occur via retrotransposition. This is also in contrast to duplication of genes via unequal crossing over, which results in tandem copies. These insights will require considerably more analysis for a definitive resolution, but it seems that these ncRNAs may not use the same duplication and/or translocation mechanisms as protein-coding genes.

Development of a protein-coding gene set

An evidence-based system (Ensembl³⁶) and two comparative gene prediction methods (Twinscan³⁷ and SGP-2 (ref. 38)) together predicted a common set of 106,749 protein-coding exons, with 85,929 additional exons predicted by one or two methods (Supplementary Table S3). Particular attention was paid to the identification of selenoproteins, which are usually mispredicted in annotated genomes because of their usage of the TGA codon, usually a stop codon, to code for the amino acid selenocysteine (see Methods). Of the human genes predicted using chicken as the "informant", only 311 genes predicted by SGP-2 are absent from previously identified sets (namely, Vega⁴⁰, Ensembl⁴¹, RefSeq⁴², MGC⁴³ and H-Invitational⁴⁴) and have homologous chicken predictions that possess orthologous intron positions. These data, and those of another study (E. Eyra *et al.*, unpublished data), suggest that most of the protein-coding genes conserved among vertebrates are represented in existing complementary DNA sets.

We tested the sensitivity and specificity of the chicken gene predictions. Sensitivity was assessed by comparing predicted exons to those of chicken cDNAs³² representing long open reading frame (ORF)-containing protein-coding genes (Table 3). All three methods correctly predicted about 80% of cDNA-based exons with >80% coverage. An independent SAGE-based analysis (ref. 166, and M. B. Wahl *et al.*, unpublished data) provided a similar, although marginally lower, estimate. Specificity was assessed by testing random exon pairs from the prediction sets using polymerase chain reaction with reverse transcription (RT-PCR) (E. Eyra *et al.*, unpublished data, and ref. 44). Briefly, Ensembl predictions

Table 2 Families of ncRNA genes in the chicken genome

RNA type	Number in chicken	Number in human	Chicken in synteny*	Conserved synteny†	Function
tRNA	280	496‡	158	52 (33%)	Protein synthesis
5S rRNA	12	301	4	0 (0%)	Protein synthesis
5.8S rRNA	3	9	1	0 (0%)	Protein synthesis
18S rRNA	0	0‡	—	—	Protein synthesis
28S rRNA	0	0‡	—	—	Protein synthesis
U1	18	146	45	9 (20%)	Spliceosome
U2	6	88			Spliceosome
U4	4	119			Spliceosome
U5	9	36			Spliceosome
U6	15	821			Spliceosome
U4atac	1§	1‡			Minor spliceosome
U6atac	4§	5‡			Minor spliceosome
U11	1§	1‡			Minor spliceosome
U12	1	2			Minor spliceosome
miRNA	121	191	87	77 (89%)	Translation repression
snoRNA	83§	245‡	63	50 (79%)	rRNA/snRNA processing
RNase P	1	1	12	7 (58%)	tRNA 5'-end processing
U7	1	184			Histone mRNA 3'-end processing
SRP	3	12			Protein secretion
7SK	4	166			Translational regulation (?)
Y	2	739			Ro RNP component
Telomerase RNA	1	1			Telomerase
BIC	1	1	—	—	Unknown

ncRNA genes were predicted as described in the Methods, except where indicated. Some human gene counts include significant numbers of pseudogenes.

*The number of chicken predictions located in conserved blocks that have defined syntenic regions in human (grouped into classes).

†The proportion of chicken predictions that have a syntenic human prediction.

‡Human genome ncRNA predictions from T. Jones and S. R. Eddy (<http://ftp.genetics.wustl.edu/pub/eddy/annotation/human-hg16/>). This human set contains 7,196 ncRNAs, 6,124 of which are putative pseudogenes.

§Chicken ncRNA genes identified by homology.

Table 3 Sensitivity of gene prediction

Feature	Ensembl	Twinscan	SGP-2
Exact exon (%)	61	53	60
80% coverage exon (%)	85	77	85
Total exons	179,084	195,665	203,834

Sensitivity of gene predictions as measured by comparison to ORF-containing cDNAs. Numbers are the percentage of coding exons from the cDNA-based models found by the three prediction systems. The sensitivity numbers are quoted at two levels: exact exon prediction and >80% coverage of the cDNA exon.

have a false positive rate of ~4%. When an exon pair is predicted by any two of the three methods (predominantly joint Twinscan plus SGP-2 exons) ~50% are confirmed, suggesting that some genes are missing from the Ensembl set, but we cannot reliably distinguish these from a similarly large number of Twinscan plus SGP-2 false positives. Using our estimates of specificity and sensitivity, we predict a total of between 20,000 and 23,000 protein-coding genes in chicken, with 80–90% of these found in the present Ensembl set (see Methods). This estimate overlaps the lower bounds in the corresponding ranges for mammalian genomes determined by similar calculations (for example, see refs 2, 3, 45).

Evolutionary conservation of gene components

Alignments of chicken and human orthologous protein-coding genes demonstrate the expected pattern of sequence conservation, with highest identity in protein-coding exons and minimal identity in introns (Fig. 2). These alignments allowed us to examine sequence conservation at different sites within genes.

Alignments of coding regions often did not extend to the previously annotated human protein start codons. Rather, we observed a fourfold increase in the frequency of methionine at the first position of the alignment (Fig. 3), suggesting that these internal ATG codons could be the true start sites for at least some of ~2,000 human genes. For these proteins, the overall distribution of amino acids upstream of the end of the alignment in human was markedly different from that downstream and was more consistent with a codon distribution derived from non-coding nucleotide sequence. Using this comparative signal and other features, such as the Kozak sequence⁴⁶, we can potentially improve the annotation of mammalian protein-coding start sites.

Sequence conservation around mammalian splice sites can be predicted by divergence at unselected (non-consensus) base pairs at the neutral rate, coupled with purifying selection on sites matching the splice site consensus⁴⁷. Given the high level of neutral site divergence that has occurred between mammalian and chicken

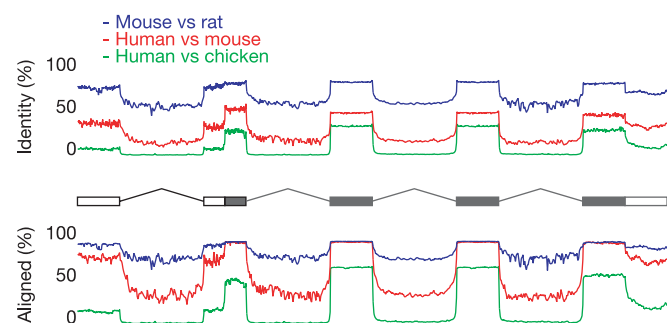


Figure 2 An idealized protein-coding gene structure showing average percentage alignment and average percentage identity (including gaps and unaligned regions) over 10,000 orthologous gene structures in either human–chicken, human–mouse or mouse–rat alignments (as aligned by BLASTZ¹⁵⁶). The reference structure was taken from human or mouse, and only those with cDNA-based definitions of the structure were used. The central figure shows an idealized gene structure, with the grey exons representing coding sequence and white boxes representing 3' and 5' untranslated regions.

orthologous sequences (see neutral evolutionary rate, below), one would expect that orthologous mammalian–chicken splice sites should show a level of conservation no different from that of any unrelated pair of splice sites. However, in contrast to analogous comparisons within the mammalian lineage, there is a detectable signal in orthologous splice site comparisons beyond the consensus derived from comparing non-orthologous splice sites (Supplementary Fig. S1). This suggests that either some subtle classes of splice site sequences are conserved beyond the generic consensus that can only be observed at the bird–mammal evolutionary distance, or that there is a significant but weak conservation in mammalian introns that is not detectable in mammalian–bird alignments⁴⁸.

To explore the role of conserved non-coding sequence segments that are probably regulators of protein-coding genes, we examined the frequency of non-coding alignments of at least 100 base pairs (bp) in, respectively, the 5' flanking region, 5' untranslated region (UTR), at least one intron, 3' UTR, or 3' flanking region (see Methods) within human–chicken orthologue pairs in relation to gene function (as determined by gene ontology (GO) category, Table 4). Some GO categories (for example, development and transcriptional regulation) showed enrichment for conservation in all five regions, suggesting that conserved regulatory signals exist within all of these locations. However, other categories showed more specific patterns. As one example, introns of ion channel genes are particularly enriched for conserved sequences, in agreement with reports that such introns contain RNA-editing targets^{49,50}.

Pseudogenes and retroposed copies in the chicken genome

Only 51 duplicates of protein-coding genes probably formed by retroposition (that is, exhibiting loss of introns)⁵¹ were identified in the chicken genome, in contrast to the more than 15,000 cases observed in mammalian genomes^{3,52}. In mammals, the ancient LINE1 (L1) transposable element is responsible for the origin of most if not all retroposed (pseudo) genes⁵³. Although birds host their own LINE-like elements (chicken repeat 1 (CR1); see below)⁵⁴, the reverse transcriptase encoded by these elements is unlikely to copy polyadenylated mRNAs⁵⁵, probably explaining the paucity of processed pseudogenes in chicken. Within the set of 51 (Supplementary Table S4), 36 clearly represent pseudogenes, because their former coding regions are disabled by alterations (including frameshifts and premature stop codons) that preclude protein function. Among the remaining 15 elements, eight show strong evidence for selective constraint (Supplementary Table S4) and therefore may represent functional retroposed genes. We found no

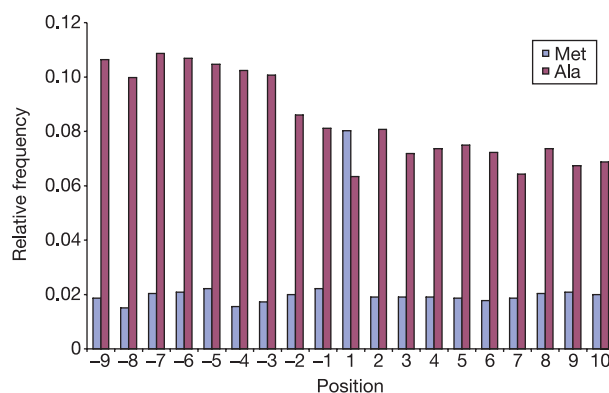


Figure 3 Histogram of amino acid distributions centred on the start of human–chicken alignments where the alignment is >30 amino acids from the putative translation start in human and less than 100 amino acids in length, using the human protein sequence. Alanine is shown as an example of non-methionine amino acids: many amino acids show significant changes before compared with after the alignment.

Table 4 Conservation near human genes

5' flank	5' UTR	Intron	3' UTR	3' flank	GO
0.99	0.95	9.7×10^{-13}	0.020	0.12	Adenyl nucleotide binding
0.99	0.96	0.0	1.7×10^{-2}	0.13	ATP binding
0.13	0.1	5.1×10^{-8}	8.4×10^{-5}	2.5×10^{-2}	Calmodulin binding
6.5×10^{-9}	1.1×10^{-6}	9.3×10^{-8}	1.1×10^{-6}	1.5×10^{-7}	Development
5.8×10^{-2}	9.9×10^{-4}	3.8×10^{-11}	8.2×10^{-4}	1.6×10^{-4}	Ion channel activity
1.0	1.0	1.1×10^{-10}	7.4×10^{-2}	0.42	Motor activity
6.0×10^{-5}	8.9×10^{-4}	1.6×10^{-5}	2.4×10^{-2}	0.10	Muscle development
2.7×10^{-3}	1.0×10^{-3}	1.7×10^{-5}	1.9×10^{-8}	6.5×10^{-9}	Neurogenesis
0.97	0.67	1.2×10^{-12}	1.8×10^{-5}	0.13	Protein metabolism
0.85	0.91	1.2×10^{-12}	6.3×10^{-4}	0.29	Protein-tyrosine kinase activity
1.4×10^{-6}	1.1×10^{-4}	5.7×10^{-4}	9.8×10^{-13}	2.3×10^{-10}	Regulation of transcription
1.2×10^{-9}	6.3×10^{-2}	1.0	1.0	0.20	Rhodopsin-like receptor activity
8.0×10^{-2}	8.5×10^{-2}	3.0×10^{-5}	1.1×10^{-4}	5.9×10^{-3}	Steroid hormone receptor activity
1.0	1.0	1.2×10^{-3}	8.7×10^{-10}	2.3×10^{-2}	Ubiquitin-protein ligase activity

Enrichment for conservation with chicken in several classes of gene-proximal regions for some GO categories of human RefSeq genes. *P*-values (that is, probabilities that enrichments as strong or stronger than the observed ones may occur by chance alone; see Methods) are shown.

clear bias towards either particular gene families or chromosomal locations for the retrocopies (Supplementary Table S4).

Interspersed repeat content of the chicken genome

Interspersed repeats are predominantly copies of transposable elements in various stages of decay. Less than 9% of the chicken genome could be classified as such, and only 11% was annotated when satellites or any lower-copy-number repetitive DNA segments were included (Table 5; see also Methods). This is markedly lower than the 40–50% interspersed repeat density observed in mammalian genomes^{1,2}, and leaves, with coding regions comprising another 4%, over 85% (~900 Mb) of the current assembly unexplained. This large amount of unexplained genetic matter—which could primarily constitute ancient transposable elements that have mutated beyond recognition—and the high divergence (age) of many recognized repeats (Supplementary Fig. S2) suggest that the low interspersed repeat density in chicken is due to low (recent) transposable element activity rather than to a high deletion rate.

Most of the interspersed repeats are CR1 LINE copies

A single type of non-LTR (long terminal repeat) retrotransposon or LINE, CR1 (refs 54, 56, 57), comprises over 80% of all interspersed repeats in the chicken genome (200,000 copies). CR1 resembles the mammalian L1 element in having a (G+C)-rich internal promoter region, followed by two ORFs. A full-length CR1 element is 4.5 kb, but all but 0.6% of the CR1 copies are truncated from their 5' end. As sequences near the 5' end are needed for retrotransposition, the success of CR1 in the bird genome seems remarkable. Comparison of the length distribution of CR1 and L1 copies (Supplementary Fig. S3) suggests a higher efficiency of the L1 reverse transcriptase or higher stability of the L1 transcript⁵⁵. CR1-like elements, unlike L1 elements, do not create target site duplications, probably explaining the absence of copies with 5' inversions so common for L1 (Supplementary Fig. S3). It is unclear whether CR1 is currently active in the chicken. We found only one full-length element with

intact ORFs in the chicken genome: a copy at chromosome 6 (GGA6; 661,970–666,111) that is >2% divergent from the CR1-F consensus or any other element. However, long, minimally divergent CR1 copies are often interrupted by sequence gaps in the present assembly, and a still-active CR1 source gene could have been collapsed and thus missed.

We reconstructed 11 complete CR1 source genes from the copies in the chicken assembly, although the abundance of 3' end fragments allowed further division into 22 subfamilies. Figure 4 shows the phylogenetic relationship of the 11 source genes based on the alignment of their ORF2 product. The evolution of CR1 in the bird genome seems to differ from that of L1 in mammals, in that several widely divergent elements have been active in parallel, whereas in mammals a single lineage of L1 has been dominant^{58,59}. The most recently active CR1 elements in chicken (CR1-F and CR1-B) are less than 70% identical over their ORF2 coding region, whereas the human and mouse L1 ORF2 products are 78% identical. This high divergence between subfamilies probably led to earlier estimates of only 30,000 (ref. 57) and 100,000 (ref. 55) copies, and the current estimate of 200,000 copies is also probably low. The location of the turtle CR1 element in the ORF2-based phylogenetic tree suggests that the main branches of chicken CR1 elements may predate the turtle–bird speciation. Otherwise the tree follows species phylogeny, suggesting that CR1 elements are ancient, vertically transmitted inhabitants of vertebrate genomes.

The most consequential difference between CR1 and L1 elements is in their 3' end structures: the L1 3' UTR has evolved with seemingly little constraint on the primary sequence except the polyadenylated tail⁵⁹, whereas the CR1 3' UTR is remarkably conserved between all derived subfamilies⁵⁵ and ends with a (ATTCTRTG)_n microsatellite in all chicken CR1 subfamilies, as well as in the turtle CR1 and the ancient L3 element. The CR1 reverse transcriptase presumably has high substrate specificity, whereas the L1 proteins are known to be highly promiscuous. All polyadenylated transcripts in mammals are potential substrates for the L1 reverse transcriptase, and mammalian genomes are littered with processed pseudogenes and SINEs that have been retroposed by the L1 machinery. As noted above, processed pseudogenes are rare in the chicken genome.

Missing SINEs

SINEs are small, non-autonomous retrotransposons derived from structural RNAs; they contain an internal polymerase III promoter and, generally, a 3' end derived from a LINE-like element in the same genome, which when transcribed is recognized by the LINE machinery for transposition. In all vertebrate and most other animal genomes studied thus far, at least one recently or currently active SINE family has been found, mostly derived from a transfer RNA and often constituting the most numerous interspersed repeat. It is therefore remarkable that the abundant chicken CR1 element

Table 5 Composition of interspersed repeats in the chicken genome

Repeat type	Copy number	Density				
		Overall (%)	Macro (%)	Micro (%)	Z (%)	Unassigned (%)
CR1	205,000	6.4	7.4	3.2	10.4	8.0
MIRs/LINE2	10,000	0.1	0.1	0.1	0.1	0.1
LTR elements	12,000	1.3	1.3	0.5	1.8	3.3
DNA transposons	13,000	0.8	1.0	0.3	1.5	0.8
Simple repeats	12,000	0.7	0.6	0.4	0.7	1.5
Satellites	2,000	0.1	<0.1	<0.1	<0.1	0.9
Total	254,000	9.4	10.2	4.5	14.5	14.5

'Macro' represents the five largest chromosomes; 'Micro' represents chromosomes smaller than and including chromosome 12; 'Z' is the male sex chromosome; and 'Unassigned' are sequences unassigned to a chromosome.

does not seem to be associated with a single SINE, especially as the closely related CR1 element in turtles⁶⁰ and L3 in ancestral mammals are or were paired with SINEs. There are about 10,000 faint matches in the chicken genome to MIR and MIR3 (the SINEs associated with L2 and L3, respectively), which originated before the mammal–bird speciation, but unlike in mammals, no new SINEs seem to have replaced these ancient elements upon their extinction.

Retrovirus-like elements

As in mammalian genomes, all retrotransposons with LTRs in the chicken genome belong to the vertebrate-specific class of retroviruses, whereas no copies of gypsy or copia retrotransposons were found. We reconstructed 14 internal and 41 LTR sequences for endogenous retroviruses (ERVs) or their non-autonomous companions, with representatives of all three recognized subclasses (class I to III). The assembly contains one of the two copies of the avian sarcoma/leukosis virus provirus known to exist in the sequenced genome (see Methods). The class II chicken GGERVK10 and class III GGERVL endogenous retroviruses may still be active, as their copies in the chicken genome are less than 3% diverged. We reconstructed several subfamilies of the most abundant ERV, GGERVL, which is most closely related to the mammalian ERV-L⁶¹. Considering the long descent of ERVL subfamilies in mammals⁶², it is tempting to propose that GGERVL and its mammalian relatives have been endogenous in both lineages since the mammalian–bird split. However, all GGERVL subfamilies are young (between 0% and 13% divergence), and the nature of

retroviruses suggests that independent introduction in the germ line is a valid possibility as well.

DNA transposons

Only two ancient DNA transposon families were found in the chicken genome: the unrelated activator-like Charlie12_GG and mariner-like GGMR elements. Thus, like that of mammals^{1,63}, the bird germ line may be protected from infiltration by DNA transposons. Both Charlie12_GG and GGMR left copies that are now ~16% diverged from the consensus, and appear to have been active contemporaneously. This is apparent from a hybrid interspersed repeat consisting of a GGMR copy within a Charlie12_GG element, from which we infer that the mariner-like element must have transposed into the activator-like element when the latter was still active.

Protein content evolution of chicken and mammalian genomes

Conservation of vertebrate domain and protein content

About 60% of chicken protein-coding genes have a single human orthologue (Fig. 5); for the remainder, orthology relationships are more complex or are not detectable. Chicken and human 1:1 orthologue pairs exhibit lower sequence conservation (median amino acid identity of 75.3%) than rodent and human 1:1 orthologue pairs³ (~88%), as expected (Fig. 6a). Orthologous sequences involved in cytoplasmic and nuclear functions are more conserved than those implicated in reproduction, host defence and adaptation to the environment (Fig. 6b). Sequence conservation of expressed chicken genes, in comparison to their human orthologues, is non-uniformly distributed among chicken tissues. Sequences expressed in the chicken brain, as indicated by EST data, are more conserved than testis-expressed sequences (Fig. 6c). Moreover, genes with ESTs from few (<4) tissue types are significantly ($P < 0.001$) less

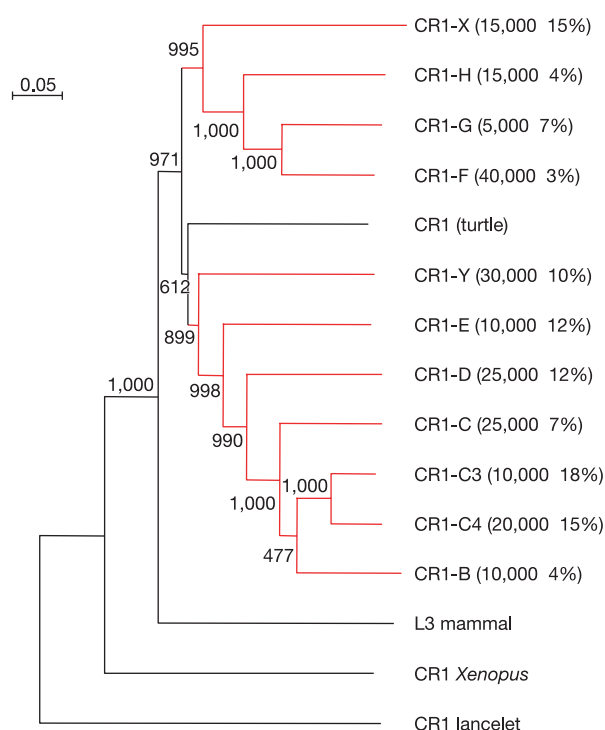


Figure 4 Neighbour-joining tree showing the phylogenetic relationship of the 11 major chicken CR1 subfamilies, CR1 in the *Platemys spixii* turtle (GI:2317255), the *Branchiostoma floridae* lancelet (GI:17529693), *Xenopus laevis* and the ancient mammalian L3 (ref. 160), based on the multiple alignment of the ORF2 products (we derived consensus sequences for the chicken and *Xenopus* CR1s and L3 with complete ORF2 products). Chicken lineages are indicated in red. Bootstrap values are indicated, as well as (in parentheses) the observed copy number of each subfamily and the average substitution level of the copies compared to the consensus. The nomenclature for chicken CR1 is an extension from ref. 161, which defined the subfamilies CR1-A to CR1-F on the basis of the 3'-end fragments.

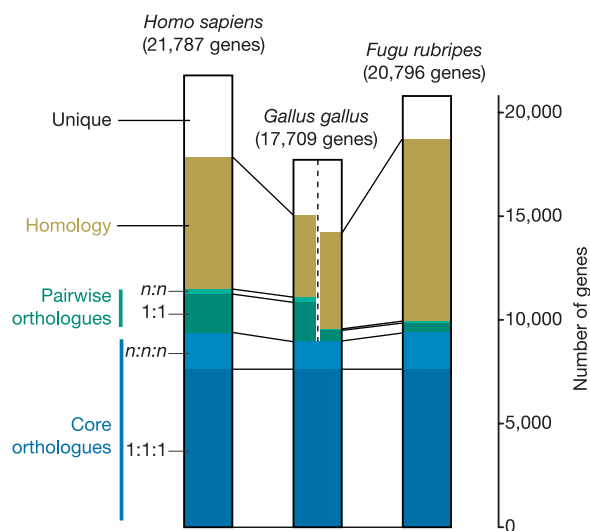


Figure 5 Chicken genes classified according to their predicted evolutionary relationships with genes of two other model vertebrates (*Fugu* and human). Forty-three per cent of the chicken genes are present in 1:1:1 orthology relationships for the three species. Also present in three species are *n:n:n* (many:many:many) orthologues; putative gene duplication events have resulted in multiple genes in at least one of the species. Pairwise orthologues are assigned when orthology is not detectable in the third species. Between *Fugu* and chicken, pairwise orthologues are rare (as expected), and might be indicative of gene loss in the lineage leading to humans. For a substantial number of genes, clear orthology relations cannot be described at all, but some similarity to genes in the other species remains detectable ('Homology', E -value cutoff is 10^{-6} in Smith–Waterman searches at the protein level). See Methods for details of orthology assignment.

conserved than sequences with ESTs from many (>6) tissues (median identities of 68.3% and 76.0%, respectively). The latter finding mirrors previous results for mammalian genes^{64–66}.

Genes that are conserved between human and chicken are often also conserved in fish: 72% (7,606) of chicken–human 1:1 orthologues also possess a single orthologue in the *T. rubripes* genome⁶⁷. These genes represent a conserved core that is likely to be present in most vertebrates (Fig. 5). Their sequences are more conserved than other chicken–human 1:1 orthologues (Fig. 6a), indicating that they have been subjected to a greater degree of purifying selection. Chicken, human and pufferfish genes also encode a similar set of protein domains. Of 1,085 Pfam and SMART domain families in InterPro^{68–70} that are encoded in at least three chicken genes, only two families are unrepresented among human genes, and a further 21 families are absent from *Fugu* genes.

Using a manual approach combining synteny information and highly sensitive search parameters, we were able to identify chicken orthologues of several immune-related genes previously

thought to be specific to mammals. Owing to high sequence divergence (Fig. 6b), these were not predicted during the automated gene build process. These include the antimicrobial protein cathelicidin, colony-stimulating factor, leukaemia inhibitory factor, interleukin-3 (IL-3), -4, -7, -9, -13 and -26, and three secretoglobins. *IL-26* was previously known only in humans (it is a pseudogene in mouse and rat⁷¹), thus chicken represents the only available model organism with which to investigate *IL-26* function.

Gene and family differences

We next sought to identify those protein and domain families that are over- or under-represented in chicken, compared with mammals (Table 6 and Fig. 7). However, this type of analysis can be complicated by artefacts inevitably present in a draft genome project such as those introduced by gaps in sequencing coverage, assembly, or gene prediction. In chicken, although many are present in partial, fragmented form within the genome assembly, we estimate that roughly 5–10% of genes are substantially truncated

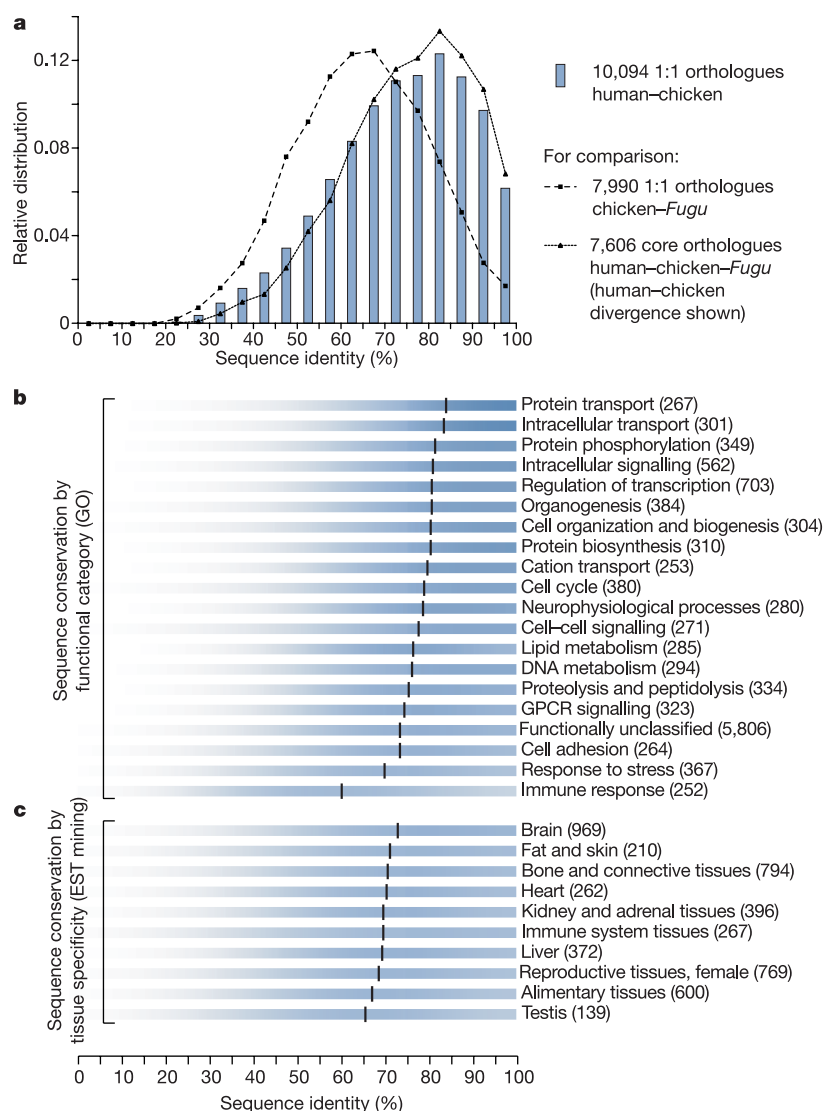


Figure 6 Sequence identity of orthologues. **a**, The percentage amino acid identity distribution of 1:1 orthologues between human and chicken, between chicken and *Fugu*, and between human–chicken orthologues that are also present in 1:1 relationships with *Fugu*. **b**, The percentage amino acid identity distribution of human–chicken 1:1 orthologues broken down by functional categories according to the GO subtree 'biological process'. Genes assigned to multiple categories were counted more than once. Vertical

bars indicate the medians of the distributions. **c**, The percentage amino acid identity distribution of chicken–human 1:1 orthologues broken down by tissue category. Vertical bars indicate the medians of the distributions. Female reproductive tissues include utero-vaginal, ovary and oviduct; immune system tissues include spleen, thymus, caecal tonsil and bursa of Fabricius; and alimentary tissues include gizzard, stomach, and large and small intestines.

in or missing from the Ensembl gene set (see Methods). Nonetheless, selecting our approaches with this in mind there are many insights to be gained, particularly in those areas not affected by these issues such as gene losses in human and gene innovations and expansions in chicken. Here, we document gene innovations, losses and expansions in chickens and mammals, and the evolution of function in orthologues.

Gene innovations in chicken. Expansion of and innovation within gene families in different lineages are often correlated with divergent phenotypes. For example, scales, claws and feathers of birds are formed using an avian-specific family of keratins, whereas hair fibre formation in mammals involves a distinct keratin family, which has greatly expanded within this lineage (Fig. 7). Of the ~150 avian keratins identified in the chicken genome, 30 are found in tandem arrays on GGA27. We also considered proteins that are specific to the eggshell, such as ovocleidin 116, for which homologues were not previously known outside of birds. However, with further analysis, we predict that ovocleidin 116 has mammalian orthologues, namely primate matrix extracellular phosphoglycoprotein (Blastp, $E = 2 \times 10^{-4}$), indicating that, despite low sequence identity (35% over 80 amino acids), both avian and mammalian genes perform similar roles in calcification. Gene innovation may also occur through domain accretion¹. These events are extremely rare among mammalian genes⁷², and we succeeded in detecting only a single instance within birds for a gene (ENSGALG00000000805) that encodes both scavenger receptor cysteine-rich (SRCR) and fibrinogen-related domains. A single exon, encoding the SRCR domain, appears to have been inserted into this gene (Supplementary Fig. S4) relatively recently, because comparison to its paralogue (ENSGALG00000000732) shows considerably fewer synonymous substitutions (K_S value of 0.034) than do the majority of chicken–human orthologue pairs (median K_S value of 1.66).

Genes absent from chicken. Genes encoding vomeronasal receptors, casein milk proteins, salivary-associated proteins (statherin and histatins) and enamel proteins seem to be absent from the chicken: from within both the EST sets and the genome. This is unlikely to result from imperfections in the chicken genome assembly because it preserves orthologues of closely linked (syntenic) mammalian genes. These absences might therefore mirror the evolution of the vomeronasal organ and mammary glands in mammals, and the loss of teeth in birds. The presence in fish of enamel-associated genes and their absence in chicken, together with absence of chicken casein and salivary-associated genes, which all cluster together in mammalian genomes, is consistent with a previous suggestion⁷³ that these have all descended, by gene duplication and rapid sequence diversification, from a common ancestor, possibly an enamel-associated gene.

Loss of an entire domain family in the protein repertoire is unusual. Nonetheless, the SCAN domain family seems to be unrepresented in the chicken gene set and genome. SCAN domains are dimerization motifs that are found, often with zinc finger domains, in more than 60 human proteins^{74,75}. In *Fugu* and zebrafish, a total of three SCAN domains are found, not associated with zinc fingers but instead within large retrovirus-like proteins. This phyletic distribution indicates a possible acquisition of new

function in the synapsid or mammalian lineages, whereas in the avian lineage the domain might have remained associated with retroviral sequences that died out subsequently.

Overall, we find that among the predicted chicken genes there is a notable under-representation of genes that are widely conserved and were presumably present in the mammal–bird common ancestor. We estimated gene innovation and loss by considering patterns of the presence or absence of orthologous genes for nine metazoan genomes, and by reconstructing the most parsimonious gene contents of ancestral root species, using the plant *Arabidopsis thaliana* as an outgroup (Fig. 8; see also Methods). Despite uncertainties stemming from the incompleteness of current genome sequences, all metazoan lineages seem to be gaining orthologous core genes, with the notable exception of the ancestor of the Diptera^{76,77}. Among the vertebrates, the chicken seems to be the only species thus far sequenced to have lost more of those genes than it has gained over an extended period of time. At present, it is difficult to estimate to what extent this observation is influenced by gaps in the assembly. Indeed, 57% of the apparent losses that are otherwise conserved as single copy genes in mammals and insects are represented in chicken EST libraries (reciprocal best match, bitscore ≥ 200). However, this coverage is lower than the average for this gene class (88%), suggesting that not all of the absences can be attributed to assembly gaps. There are a number of potential explanations for these data. The most straightforward is that some of these core genes were deleted within the avian lineage. An alternative is that they have experienced accelerated evolution. Targeted investigations will be required in order to resolve whether birds have lost unusually large numbers of genes that are otherwise conserved among metazoa.

Expansion of domains and gene families in chicken. Three domain families are significantly over-represented in chicken with respect to human (Fig. 7). The most notable case is an apparent 40% expansion of SRCR domains. Many of these domain sequences are similar to those of human *DMBT1*, a proposed regulator of mucosal homeostasis⁷⁸. This suggests a link between the abundance of SRCR domains in chicken and its adaptive requirements in mucosal defence.

To gain a more in-depth view of family expansions in chicken we identified, from the set of automatically derived orthologues, nine chicken gene families containing at least fourfold more representatives than in human (Fig. 7). Most of these families seem to have roles in immunity and host defence. Family sizes were further refined by re-predicting genes and pseudogenes from chicken and human assemblies using Exonerate (G. Slater and E. Birney, unpublished software) and Genewise⁷⁹ (Table 7). The most marked expansion involves at least 218 non-identical chicken genes that are predicted to be orthologous to one of two olfactory receptor genes (*OR5U1* and *OR5BF1*). In humans, *OR5U1* and *OR5BF1* genes lie within olfactory receptor gene clusters that each are positioned next to paralogous major histocompatibility complex (MHC) class I gene clusters⁸⁰. These olfactory receptors have been tentatively proposed to be involved in odorant-mediated detection of MHC diversity⁸¹. Duplication of and/or gene conversion within *OR5U1*-like and *OR5BF1*-like genes in chicken appear to have been

Table 6 Changes to the genome content of chicken and human

Species	Probable innovation	Change in number	Loss
Changes to domains			
Birds	Avian-specific feather keratin domain	SRCR domain: over-represented in chicken	SCAN dimerization domain
Mammals	Mammalian hair keratin domains	Intermediate filaments: over-represented in mammals	Avidin domain (egg-white)
Changes to genes			
Birds	Gene with novel domain combination: fibrinogen-related and SRCR domains	Olfactory receptor type 5U1/5BF1: expansion in chicken	Enamelin and amelogenin
Mammals	Caseins (milk proteins)	Mammalian taste receptors: expansion in mammals	DNA photolyase (nocturnal lifestyle)

Interpretation of presence and absence patterns as well as family size variation between chicken and human. For details on the method of detection see Methods.

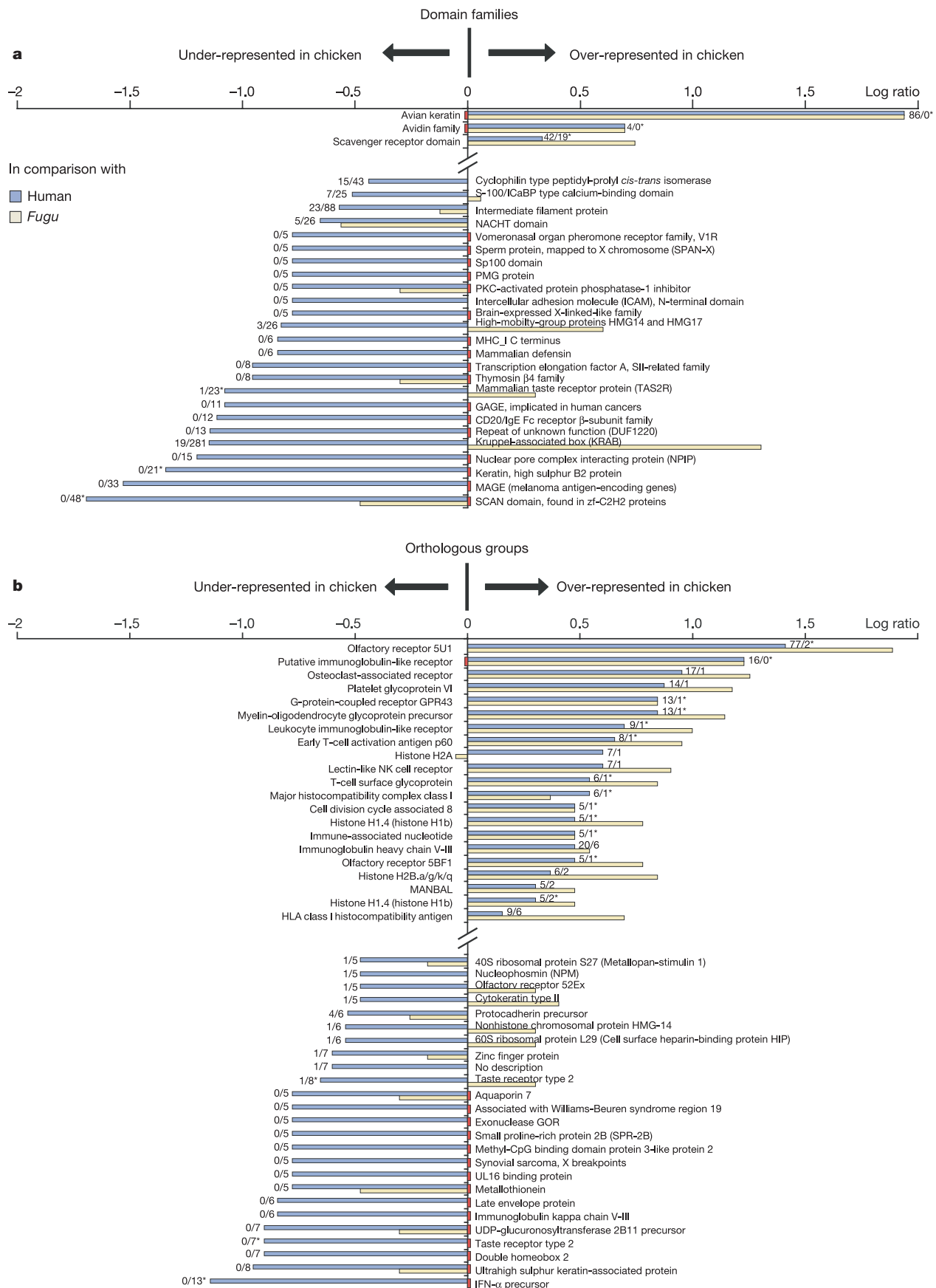


Figure 7 Loss, innovation, expansions and contractions of protein families: domain counts and orthologous relations. All at-least-twofold over- and under-representations (separated by a solidus) are shown for both members of domain families (**a**) and 'many to many' orthology relations (**b**). Ranking of families and groups has been done with respect

to the human genome; *Fugu* data are also shown for comparison. An asterisk indicates that manual analyses refined what were otherwise automatic counts. Families not subjected to twofold variations are not shown.

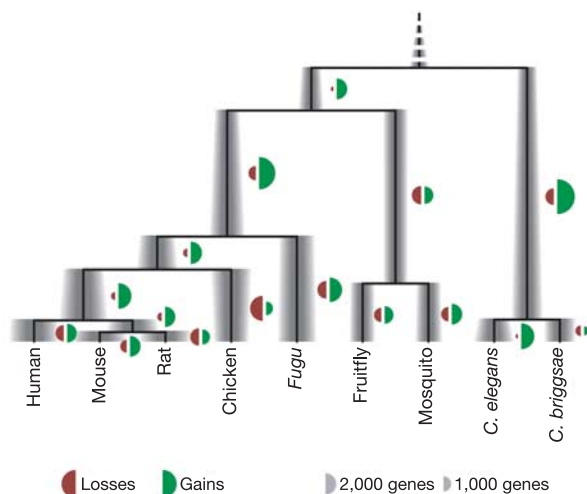


Figure 8 Approximate history of animal genomes. The gene content of ancestral animals—as estimated by using automatically delineated orthologous groups (see Methods)—assuming parsimony as well as a lack of horizontal gene transfer among animals. The inferred number of lost (and gained) genes on each lineage is shown as a half-circle, the area of which is proportional to the number. The shadings under the trees give a rough indication of the number of genes per genome. Wherever necessary, the *Arabidopsis* genome was used as an outgroup to infer the direction of changes. Ancestral estimates close to the root are likely to be underestimates because of unrecognized orthology relations and putative ancestral genes failing to survive in any of the extant genomes. The gene count of present-day genomes was approximated by considering only genes with orthology support; any remaining genes were considered only if they had substantial similarity support within the genome (to avoid spurious gene predictions, pseudogenes and/or fragments; see Methods).

both extensive and relatively recent in this lineage (Supplementary Fig. S5).

The *OR5U1/OR5BF1*-like genes contribute the majority of at least 283 olfactory receptor genes in the current chicken assembly, a similar number to that found for humans⁸². The large size of the olfactory receptor family seems to run counter to the textbook view (for example, see ref. 83) that birds have a poor sense of smell⁸⁴. Individual chicken researchers, however, had already suspected that chickens are not particularly anosmic (for example, see ref. 84). On five chicken chromosomes, these olfactory receptor genes are present in subtelomeric clusters, which, by analogy with human olfactory receptor clusters⁸⁵, may be associated with their rapid evolution⁸⁶. As with these genes in mammals, chicken olfactory receptor genes are interspersed with many (~100) homologous pseudogenes.

Gene innovations in mammals. The largest mammal-specific gene family is the high/ultrahigh sulphur hair keratins (also named keratin-associated proteins). Thirty-seven copies have been reported on HSA17q12-21 (ref. 87) embedded in the larger type I keratin cluster, which is present also in other mammals⁸⁸. Another

large orthologous family with multiple members in human, but none in chicken, is a subgroup of the α -interferons (Fig. 7). The expansion and diversification of interferons into various subfamilies is thought to be a mammalian innovation in response to different pathogen challenges^{89,90}. Some highly diverged interferon homologues have been found outside mammals (for example, see ref. 89), and the distant relatives in chicken and *Fugu* appear to have duplicated independently from the mammalian radiation.

Gene losses in mammals. The chicken genome enables increased precision in dating gene losses in the lineage leading to humans: we can identify losses that happened within the synapsid lineage, but before the mammalian radiation (an interval of ~110–310 Myr ago). Only one loss affects an entire domain family, the avidins, which are represented in the chicken but not in sequenced mammalian genomes (Fig. 7). Avidins are present in oviparous vertebrates as minor egg-white proteins, and closely similar homologues in zebrafish, sea urchin and bacterial genomes indicate a loss of this domain family in mammals. Mammals also seem to have lost genes encoding vitellogenin I and II, which are yolk storage proteins providing nutrients to the early embryo in the egg. These losses were probably concordant with reduction in egg size and with internalization of the embryo during mammalian development. Other mammalian losses appear to reflect a presumed episode of nocturnal lifestyle in early mammalian history⁹¹. Apart from the known loss of CPD-photolyase, which is a DNA-repair enzyme dependent on light energy⁹², we find that mammals appear to have lost at least one pigmentation gene, indigoidine synthase A, whose bacterial homologue is an enzyme involved in generating blue colour pigments⁹³.

Gene expansions in mammals. The largest mammalian expansion observed at the domain level involves the Kruppel-associated box (KRAB) domain, whose presence coincides with C2H2 zinc fingers in transcription factors. Whereas the KRAB domain has been found in more than 400 human genes⁹⁴, the chicken seems to have fewer than 140. The complete absence of this domain in *Fugu* underlines the marked expansions of this family in mammals and to a lesser degree in birds. Orthology analysis identifies an apparent gene expansion of G-protein-coupled taste receptors in mammals (Fig. 7a, b). These fast-evolving receptors were previously studied only in mammals^{95,96}. In the chicken genome, we find similar numbers of type I receptors (those responsible for sweet and umami taste; that is, certain amino acids such as Glu and Asp, and related compounds) but only three type II receptors, compared with around 30 in mammals. Type II taste receptors are thought to be responsible for the sensing of bitter tastants^{97,98}, an adaptive avoidance system that may be more variable than the other taste systems. This might indicate that birds have a limited capacity for bitter taste, or that they have recruited other G-protein-coupled receptor subtypes for sensing bitter compounds.

Gene duplication events in chicken and human. To understand gene repertoire differences between avian and mammalian lineages, we quantified the number of gene duplication events since the divergence of chicken and human lineages by examining species-specific duplicate pairs (paralogues that reciprocally have their best

Table 7 Details of family expansions in chicken

Number of chicken genes	Number of chicken pseudogenes	Chicken:human gene ratio	Annotation
202	16	202:1	Olfactory receptors 5U1/5BF1
25	6	25:1	Immunoglobulin-like receptor CHIRs
14	3	14:1	Major histocompatibility complex, class I-like sequence
13	0	13:1	Myelin-oligodendrocyte glycoprotein; immunoglobulin V-region-like B-G antigen
26	1	26:3	G-protein-coupled receptor 43; free fatty acid activated receptor 2 in leukocytes
8	0	8:1	Early activation antigen CD69
6	0	6:1	T-cell surface glycoprotein CD8 α -chain
5	0	5:1	Immune-associated nucleotide 6-like; guanosine triphosphatase

In-depth analysis of gene families that have been expanded at least fourfold, as revealed by automatic orthology analysis.

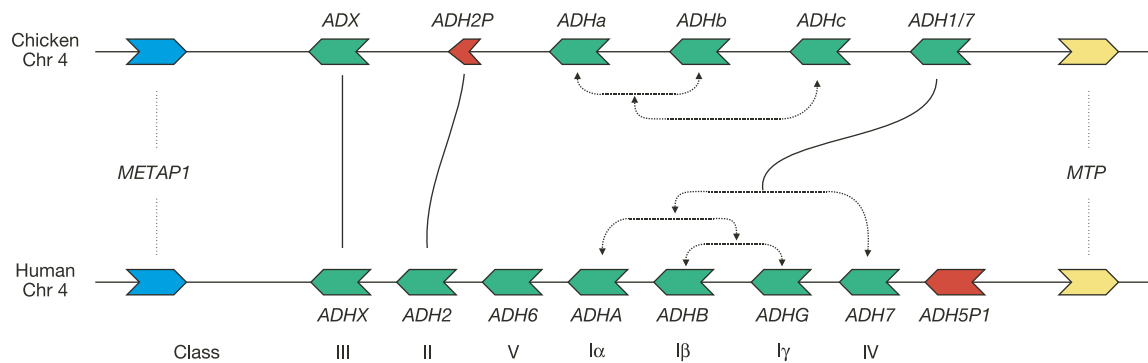


Figure 9 Evolution within the orthologous alcohol dehydrogenase (ADH) gene clusters of similar size in chicken and human. Architecture of the ADH clusters in human (HSA4q23, between positions 100.3 and 101 Mb) and chicken (GGA4, between 60.4 and 60.5 Mb). Both ADH regions are flanked by orthologous genes: *METAP1* (upstream, blue) and *MTP* (downstream, yellow). Gene names and classes used here for the eight human ADH genes are in accordance with a recent nomenclature proposed for this protein family⁹⁹. Using the human ADHX protein as homologous template, we have identified up to six ADH gene

copies in the orthologous chicken region: five are complete and one is fragmented and likely to be an *ADH2* pseudogene representing a niche loss. Only chicken proteins encoded by genes *ADHa* and *ADH1/7* had been previously identified^{162,163}. Known genes or complete predictions with no truncations are displayed in green, whereas incomplete or truncated copies (pseudogenes) are in red. The probable correspondences between the chicken and human genes (shown as lines connecting both clusters) are revealed by the neighbourhood-joining tree of the proteins shown in Supplementary Fig. S6.

hit in the same genome). Paralogues exceeding 95% pairwise identity were excluded from both data sets to avoid chicken genome assembly artefacts. In chicken, 13% (221 out of 1,712) of duplicate pairs have their closest hit in the chicken genome. In contrast, 27% (647 out of 2,426) of paralogues with <95% identity from the human genome are human specific, a pattern that is significantly different compared with that seen in the chicken (Fisher's exact test, $P < 10^{-15}$). Thus, despite similar numbers of older duplications in both genomes (1,779 in humans versus 1,491 in chicken), there have been significantly fewer paralogues specific to the chicken lineage.

Evolution of function in orthologous gene clusters

Five alcohol dehydrogenase (ADH) genes plus one pseudogene fragment are present on GGA4, whereas seven genes (plus one pseudogene) are found in the orthologous segment on HSA4p23 (ref. 99) (Fig. 9; see also Supplementary Fig. S6). The *ADH2* gene appears only recently to have been silenced in chicken because it is still functional in the ostrich¹⁰⁰. Two recent duplication events have given rise to three chicken ADH genes that might compensate for some of the lost *ADH2* functionality. These duplications, and others in human and other vertebrate lineages⁹⁹ (Supplementary Fig. S6), might indicate changes in expression patterns or even reveal episodes of adaptation to nutritional and detoxification requirements involving changes to limited gene repertoires.

Change of function in orthologues

For the past 40 years, avian species have been described as lacking a functional urea cycle because of the absence in the chicken liver of the enzymatic activity that initiates the cycle: carbamoyl phosphate synthetase 1 (*CPS1*)¹⁰¹. This observation has been linked to a difference in excretion of nitrogenous waste: whereas mammals excrete urea, birds excrete uric acid as a possible stratagem in reducing the build-up of soluble urea during development in the egg. Contrary to these expectations we have identified a full-length, apparently functional, chicken *CPS1* gene on GGA7 in the orthologous location to the human *CPS1* gene (Supplementary Fig. S7a). Characterization of the expression profile of this candidate gene revealed expression in brain and muscle, and in immune tissues (Supplementary Fig. S7b). These data, and the presence of all the other enzymes of the mammalian urea cycle in the chicken genome (data not shown), indicate that this cycle is intact in birds but might perform a function distinct from the generation of urea for excretion.

Exploring chicken genome architecture

The assembly of the chicken genome, with its distinctive karyotype and smaller size, provides an opportunity to explore important issues in genome structure and evolution. Cytogenetic, molecular and mapping data have suggested previously that microchromo-

Table 8 Comparison of properties of mammalian (A+T)/(G+C)-rich regions with chicken macro- and microchromosomes

Characteristic	(A+T)-rich	(G+C)-rich	Macrochromosomes	Intermediate/microchromosomes
Ultrastructure				
Cytogenetic band type	G-band	R-band	G-band	R-band
DNA sequences				
Gene density	Low	High	Low	High
Intron size	Longer than (G+C)-rich	Shorter than (A+T)-rich	Longer than intermediate/microchromosomes	Shorter than macrochromosomes
G+C content	Lower than (G+C)-rich	Higher than (A+T)-rich	Lower than intermediate/microchromosomes	Higher than macrochromosomes
CpG island density	Less than (G+C)-rich	More than (A+T)-rich	Less than intermediate/microchromosomes	More than macrochromosomes
Long interspersed repeats	More than (G+C)-rich	Less than (A+T)-rich	More than intermediate/microchromosomes	Less than macrochromosomes
Short interspersed repeats	Less than (G+C)-rich	More than (A+T)-rich	Almost none	Almost none
Mutation				
Synonymous rate (K_S)	—	—	Lower than intermediate/microchromosomes	Higher than macrochromosomes
Non-synonymous rate (K_A)	—	—	Same	Same
K_A/K_S ratio	—	—	Higher than intermediate/microchromosomes	Lower than macrochromosomes
GC3	—	—	Lower than intermediate/microchromosomes	Higher than macrochromosomes
Function				
DNA replication	Later than (G+C)-rich	Earlier than (A+T)-rich	—	Mostly early
Meiotic recombination rate	Lower than (G+C)-rich	Higher than (A+T)-rich	Lower than intermediate/microchromosomes	Higher than macrochromosomes
Methyl-C content	Lower than (G+C)-rich	Higher than (A+T)-rich	Lower than intermediate/microchromosomes	Higher than macrochromosomes

somes are (G+C)-rich, CpG-rich and gene-rich^{102–104}, exhibit features that are correlated with transcriptionally active DNA^{105–110}, and have structural counterparts in the (G+C)-rich mammalian chromosomal R-bands (Table 8). Our current analysis shows that the different size classes of chicken chromosomes exhibit a number of correlated attributes. Although these trends are well documented in mammals (for example, see refs 1, 2, 111–113), the sequence of the chicken genome assembly demonstrates levels of complexity, breadth and resolution that previously could not be achieved.

Comparison of physical and genetic distances

A comparison of the physical distance along each chicken chromosome derived from the sequence assembly with the genetic distance between markers (based on the sex-averaged map^{25,29}; see Methods) reveals wide variation in recombination rates that has a strong negative association with chromosome length (Fig. 10a). The recombination rate varies over an eightfold range among chromosomes (2.5 to 21 cM Mb⁻¹); rates are much higher on microchromosomes (median value of 6.4 cM Mb⁻¹) than on macrochromosomes (median value of 2.8 cM Mb⁻¹). These results contrast with the narrow range and overall lower rates found in mammalian chromosomes of only 1–2 cM Mb⁻¹ for human and 0.5–1.0 cM Mb⁻¹ for the mouse^{1,2}. The increased recombination rate of microchromosomes is such that all are likely to have total genetic lengths between 50 and 100 cM, as was expected, given the obligatory chiasma per bivalent to ensure normal segregation during meiosis¹¹⁴.

Chromosomal distributions of sequence features

Our analysis of the distributions of G+C content, CpG islands, and gene density and size was restricted to chromosomes with nearly

complete sequence coverage; this excluded chromosomes GGA16, GGA22, sex chromosomes and microchromosomes smaller than GGA28 (Supplementary Table S2 and Fig. S8). We arbitrarily designated three chromosome size groups: large macrochromosomes (GGA1–5), intermediate chromosomes (GGA6–10) and 28 microchromosomes (GGA11–38). The overall G+C content declines markedly with increasing chicken chromosome length (Fig. 10b). The G+C content in macrochromosomes shows a narrow distribution, whereas for microchromosomes the distribution is broad and shifted to higher values (Supplementary Fig. S9a). Indeed, some individual chromosomes have almost no overlap in their distributions of G+C content (Supplementary Fig. S9b). These patterns differ from the considerable overlap observed in G+C content among individual mammalian chromosomes (Supplementary Fig. S9c; see also ref. 3).

Thus both G+C content and recombination increase with decreasing chromosome size. The association between G+C content and recombination may be viewed as a dynamic cycle. A possible explanation is that chromosomal regions with a high recombination rate might elevate the G+C content by “biased gene conversion”¹¹⁵. In biased gene conversion, mismatch repair within the heteroduplexes formed during recombination results in G+C-biased gene inclusion¹¹⁶. Biased gene conversion may also increase the neutral mutation rate of microchromosomes. Furthermore, studies on a wide range of species (summarized in ref. 111) have shown that (G+C)-rich DNA has an increased recombination rate. Thus, a cycle of high recombination may lead to increased G+C content, which then leads to a further increase in recombination rate until equilibrium is reached when selective forces prevent further change. This model suggests that changes in recombination brought about by a chromosome rearrangement may lead to changes in G+C content, as recently shown in mouse¹¹⁷.

In the chicken genome 48% of CpG islands (see Methods and Supplementary Table S5), often associated with promoters and other sites of regulation^{118,119}, overlap a gene. We found that ~38% of chicken CpG islands are conserved in the human genome. However, taking into account proximity to protein-coding genes and overlaps with ESTs (see Methods and Supplementary Table S5), we find that 10% of CpG islands conserved with the human genome are not near a gene. These may represent distant regulatory regions, flank undetected genes, or serve another function.

CpG island density presents a strong negative association with chromosome length, being highest on the intermediate chromosomes and microchromosomes (Fig. 10c). Similarly, gene density shows a strong negative association with chromosome length (Fig. 10c), confirming, at higher resolution, earlier studies^{102,104}. Furthermore, we find a strong correlation between the length of a gene and the size of the chromosome in which it is found, an effect that is determined largely by variation in intron size (Fig. 10d). Although exon lengths (Fig. 10d) and numbers (data not shown) do not vary significantly among chromosome types, intergenic distances do increase with chromosome size (Fig. 10e). Intron length in the chicken correlates negatively with recombination ($r_s = -0.774$, $P < 0.001$), G+C content ($r_s = -0.934$, $P < 0.001$) and gene density ($r_s = -0.961$, $P < 0.001$), as has been reported previously for other genomes^{120–123}. The proportion of a chromosome covered by interspersed repeats increases with chromosome length (Fig. 10f; $r_s = 0.973$, $P < 0.001$, for the 14 largest chromosomes excluding GGA8; $r_s = 0.940$, $P < 0.001$, for all chromosomes when excluding the outliers GGA8, GGA24, GGA27 and GGA28). Not surprisingly, repeat density correlates positively with intron and intergenic gap length, as repeats primarily exist in these locations. Repeat density correlates negatively with recombination rate, G+C content and gene density (Fig. 10). The correlation with recombination rate is also expressed in the twofold higher than expected repeat density on sex chromosome GGZ (Table 5) and an increased density with distance from the

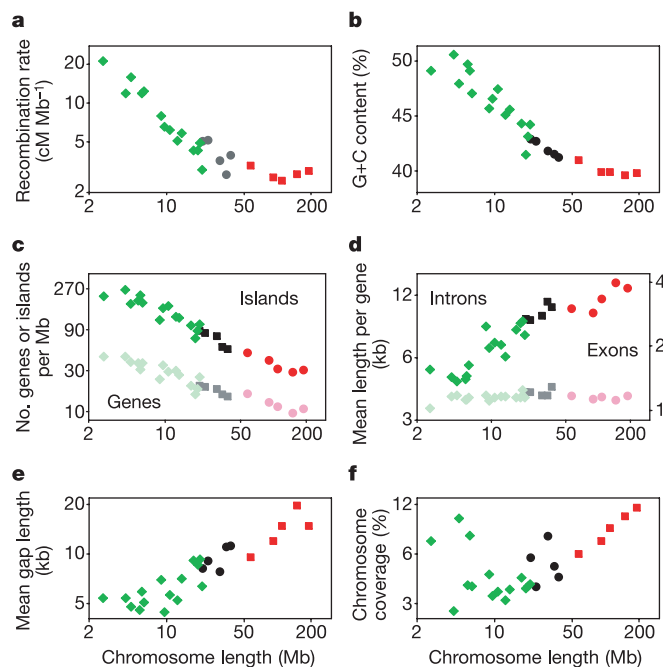


Figure 10 Relationships between chromosome sequence length and characteristics for chromosomes 1–28. **a–f**, Recombination rate (**a**), G+C content (**b**), densities of genes and CpG islands (**c**), total lengths of introns and coding exons per gene (**d**), intergenic gap lengths (**e**) and densities of interspersed repeat elements (**f**). All plots exclude GGA16 and GGA22, which have insufficient sequence, and panel **a** also excludes GGA23 and GGA25, which have insufficient genetic markers. Red, macrochromosomes; black, intermediate chromosomes; green, microchromosomes; additional paler colours indicate genes in **c** and exons in **d**.

Table 9 The short arm of chicken chromosome 4 retains its ancestral microchromosome properties

Characteristic	GGA4p	GGA4q
Physical length (bp)	18,442,167	70,692,738
Recombination rate (cM Mb ⁻¹)	4.44	2.51
G+C content (%)	42.41 ± 5.60	38.68 ± 3.36
Intron length (bp)	16,005 ± 26,850	28,503 ± 40,711
G+C content of intron (%)	46.00 ± 8.92	40.00 ± 6.22
Exon length (bp)	1,425 ± 1,089	1,567 ± 1,308
G+C content of exons (%)	51.28 ± 6.83	47.00 ± 6.26
Gene density (genes Mb ⁻¹)	19.5	10.6
CpG density (islands Mb ⁻¹)	80.96	30.64
Repeats (%)	4.84	9.13
DAPI stain	Dull, (G+C)-rich	Bright, (A+T)-rich

In some cases values are shown ± 1 s.d.

centromere (Supplementary Fig. S10). Recombination rate will influence repeat density if transposable element insertions on average are slightly deleterious and selection is more effective in regions of high recombination¹²⁴. Indeed, recently a strong negative correlation between recombination rate and repeat density was reported in primates and rodents, where recombination rate was found to be proportional to distance from the centromere¹¹¹. The correlation may be more obvious in chicken because of the larger differences in chromosome size and recombination rates. The same mechanism can be proposed to explain the correlation between the size of introns and intergenic gaps and recombination rate, assuming that longer introns and intergenic gaps are slightly deleterious.

The distinctive characteristics of microchromosomes are retained even after fusion with a macrochromosome, as illustrated by an ancestral chromosome fusion leading to GGA4. The p arm of GGA4 is orthologous to an ancestral microchromosome, as shown by comparative mapping using chromosome painting and by sequence matches with microchromosome 9 in turkey (see Methods). Interestingly, the telomeric DNA signals from fluorescence *in situ* hybridization analyses show that GGA4p possesses an interstitial telomere adjacent to the centromere (Supplementary Fig. S11). Analysis of the sequence assembly shows that GGA4p has not taken on characteristics of the macrochromosome, but rather it still has the properties of its ancestral microchromosome, including high recombination rate and high gene density (Table 9).

Synonymous substitution rates

Neutral substitution rate variation was analysed by examining synonymous substitution rates (K_S), estimated using Codeml¹²⁵, in a set of 7,529 human–mouse–chicken 1:1:1 orthologues (Fig. 11). As expected from the greater phylogenetic distance, the level of nucleotide substitution deduced from human–chicken alignments is much higher than that observed in human–mouse comparisons. The median value of K_S for 1:1 orthologues between human and chicken is 1.66, indicating that synonymous sites on average have changed one to two times in the combined lineages to humans and chickens. For the analyses presented below, we acknowledge that with such high substitution rates, the conclusions should be corroborated with data from comparisons of much more closely related taxa, in particular from two or more bird lineages.

Synonymous substitution rates are elevated in two types of

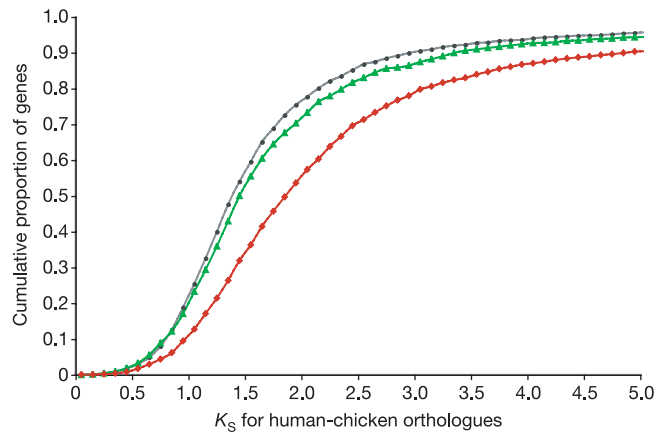


Figure 11 Genes on chicken microchromosomes possess higher synonymous substitution rates. We split the chromosomes into macrochromosomes (GGA1–5, grey circles), intermediate chromosomes (GGA6–10, green triangles) and microchromosomes (GGA11–32, red circles), and calculated their K_S values using Codeml. The cumulative distribution of genes in each chromosome size category is plotted versus the K_S values. Because large K_S values are methodologically suspect, we discarded K_S values >10.

chromosomal region. First, chicken–human K_S values are significantly higher among genes on microchromosomes (median K_S = 2.01) than they are on intermediate chromosomes (1.60) and on macrochromosomes (1.54) (Table 10; $P < 2.2 \times 10^{-16}$, Kolmogorov–Smirnov test). Despite significant differences in G+C content for the chicken chromosomes (Fig. 10b), the elevation in K_S is not entirely due to non-equilibrium base compositions of chicken or human sequences in the time since their ancestral genes diverged. Specifically, we found that K_S distribution differences between genes on chicken micro- and macrochromosomes also remain significant when only considering genes exhibiting similar G+C contents, or G+C percentage at fourfold degenerate sites¹²⁶ (Table 9). Higher neutral divergence rates are also seen on microchromosomes when genes are compared between chicken and turkey genomes¹²⁷.

Second, subtelomeric sequences, which are often duplicated and polymorphic^{86,128}, also show elevated neutral substitution rates. The synonymous substitution rates of chicken genes in regions up to 10 Mb from the ends of assembled macrochromosomes are elevated to levels that are indistinguishable ($P > 0.05$ in a Kolmogorov–Smirnov test) from those of microchromosomal genes when chicken–human alignments are considered (Table 10). It thus seems possible that the elevated K_S values associated with chicken microchromosomal genes result from the more general phenomenon of rate elevation in subtelomeres: the K_S value for a microchromosomal gene is, on average, higher than a gene on a macrochromosome because all microchromosomal genes lie within 10 Mb of one or both of their telomeres. However, it is important to note (Fig. 12) that genes on chicken microchromosomes are not, in general, subtelomeric in mammalian genomes. Nevertheless, the mammalian orthologues of chicken microchromosomal genes show significantly ($P = 0.003$) elevated K_S values even when those values

Table 10 Chicken gene synonymous substitution rates

Species	K_S microchromosomes			K_S intermediate chromosomes			K_S macrochromosomes		
	25%	Median	75%	25%	Median	75%	25%	Median	75%
Chicken–human	1.473	2.009	2.895	1.224	1.599	2.252	1.193	1.535	2.087
Chicken–human (0.48 < [GC] < 0.52)	1.409	1.867	2.657	1.172	1.479	1.876	1.196	1.556	2.088
Chicken–human (0.48 < [GC4d] < 0.52)	1.389	1.801	2.698	1.330	1.640	1.811	1.179	1.566	2.056
Chicken–human 5 Mb subtelomeric regions	1.519	2.076	3.011	1.235	1.623	2.229	1.474	2.162	3.080
Human–mouse	0.461	0.588	0.768	0.421	0.528	0.651	0.444	0.548	0.687

GC4d, G+C fraction at fourfold degenerate sites.

are derived from mouse–human comparisons. Thus, this gene set seems to have retained some characteristic other than chromosome location that has elevated neutral divergence during mammalian, as well as avian, evolution.

The overall increase in the rate of synonymous substitution for microchromosomal genes was not accompanied by significant changes in the chicken–human non-synonymous substitution rate, K_A (median values of 0.121, 0.118 and 0.116 for macro-, intermediate and microchromosomes, respectively). Microchromosomal genes on average have smaller K_A/K_S ratios (median value of 0.052, compared with 0.073 and 0.068 for macrochromosomes and intermediate chromosomes, respectively), indicating that they have been subjected to greater degrees of purifying selection. This highlights a hitherto unforeseen contribution of genomic location to coding sequence evolutionary constraint.

Segmental duplications

One unusual aspect of the human genome with respect to other sequenced genomes is the abundance (4%) of large (>20 kb), nearly identical duplications¹²⁹. In marked contrast to observations for mammalian genome assemblies^{129–132}, of the confirmed segmental duplications in chicken (see above), few exceeded 10 kb in length (Fig. 13), and none greater than 50 kb were detected. Almost all mapped duplications (93%) within the chicken genome are intra-chromosomal (excluding unplaced sequences) (Supplementary Fig. S12). Analysis of exon content of the duplicated regions revealed that only 3.7% of Ensembl predicted genes showed evidence of being recently duplicated—a slight enrichment as compared to the genome average of confirmed duplicated bases (3.0%). The proportion of uncharacterized genes within these recent segmental duplications is nearly fivefold greater than that observed for

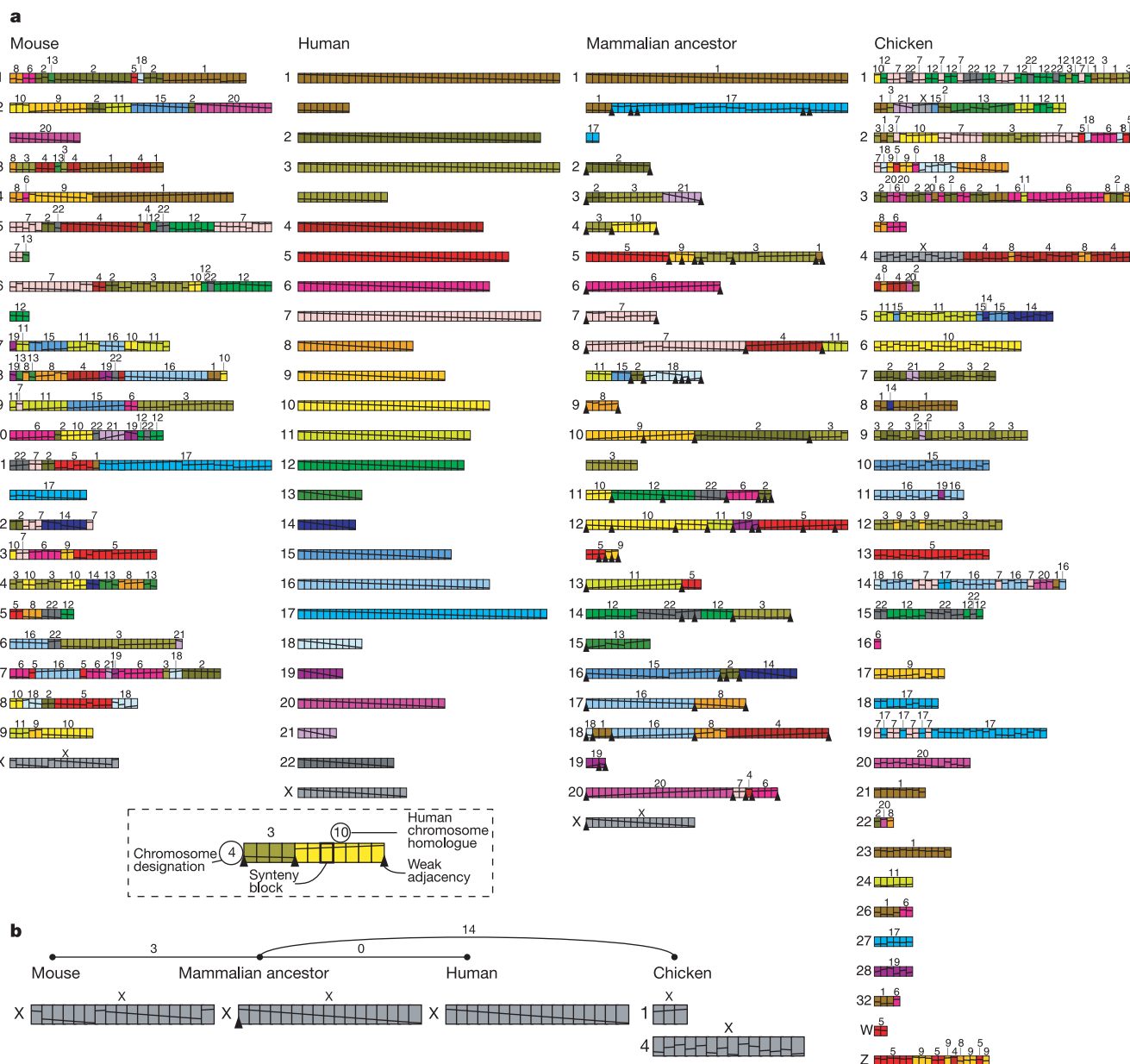


Figure 12 Putative mammalian ancestor recovered by GRIMM and MGR using the human, mouse, rat (not shown) and chicken genomes. **a**, Each genome is represented as an arrangement of 586 synteny blocks each drawn as one unit, regardless of its length in nucleotides. Each human chromosome is assigned a unique colour, and a diagonal line is drawn through the whole chromosome. In other genomes, this diagonal line indicates the

relative order and orientation of the rearranged blocks. **b**, The recovered ancestral X chromosome is optimal and unique. Gene order of the ancestral X chromosome is identical to human. Numbers associated with the lines indicate the minimum number of rearrangements required to convert between two nodes.

other unique sequences. Characterization of these may uncover lineage-specific genes important for the adaptation of the chicken.

Evolution of vertebrate genomes

Amniote chromosomal rearrangements

The evolutionary history of genome organization can be inferred from the comparative analysis of gene orders on chromosomes. Recent studies of mammalian genomes suggest a larger number of rearrangements than previously estimated^{1,2,133–136}, largely due to the underestimation of intrachromosomal rearrangements. Using the chicken genome sequence as an outgroup provides an opportunity to understand better the rate of rearrangements in mammalian lineages and the architecture of the ancestral mammalian genome.

We generated two maps of conserved synteny—defined as orthologous chromosomal segments with a conserved gene neighbourhood^{133,134,137}—among chicken, human and mouse, using DNA- and protein-level methods (see Methods). These two sets of anchors generated highly similar maps, with comparable sizes of orthologous segments and amounts of coverage of the human and mouse genomes (Table 11). We then used two approaches to quantify chromosomal dynamics: (1) by counting the number of synteny breaks where the ancestral state is confirmed by conserved synteny to an outgroup species; and (2) by estimating the minimum number of rearrangements that could explain the current genome organization. Both approaches are consistent, revealing a slow rate of rearrangement in the human lineage, about one-third that of the rodent lineage (Fig. 14). The synteny analysis using orthologous gene pairs is more sensitive at long evolutionary distances than DNA-based methods, and it allowed us to use recently sequenced fish genomes of *Fugu*⁶⁷ and *Tetraodon*¹³⁸ as outgroups. This analysis revealed an even slower rate of rearrangements in the chicken lineage (Fig. 14). The synteny maps confirm an earlier observation¹³⁹ that the human genome is closer to the chicken than to rodents in terms of chromosomal organization of genes (Supplementary Figs S13 and S14). As indicated from the analysis of the *Tetraodon* genome, this surprising level of similarity arises from an unusually low rate of interchromosomal shuffling in the lineage leading to the earliest mammal.

We reconstructed the putative mammalian ancestor genome (more precisely, the common ancestor of human–mouse–rat and all the Euarchontoglires¹⁴⁰) by looking for a scenario minimizing the

number of rearrangement events on the evolutionary tree¹⁴¹ (see Methods). Because the pairwise distances between the initial genome are substantial, it is possible to find alternative ancestors that also minimize the total number of rearrangement events. By exploring some of these alternative ancestors, we partitioned all the pairs of adjacent synteny blocks of the recovered ancestor into ‘strong’ and ‘weak’ pairs depending on whether they are present or not in all of the observed alternative ancestors (Fig. 12a). Many of the previously postulated chromosome associations of the placental ancestor correspond to strong pairs of adjacent blocks in the mammalian ancestor^{136,142}. The synteny of six human chromosomes and of ten chicken chromosomes is preserved in the mammalian ancestor, whereas synteny only of the rodent X chromosome is preserved in the mammalian ancestor¹⁴¹. This is consistent with the suggestion that interchromosomal rearrangements have been more frequent in rodents¹³⁹. The scenario recovered for the X chromosome also reveals variable rates of intrachromosomal rearrangements (Fig. 12b). There appear to have been no rearrangements between HSAX and the X chromosome of the mammalian ancestor (that is, human order is ancestral), yet there are three inversions between the mouse X chromosome (MMUX) and the mammalian ancestor X chromosome, and there are 14 rearrangements (13 inversions and 1 fusion) between the two chromosome segments in chicken (from GGA1 and GGA4) and the mammalian ancestor X chromosome.

Variation in human–chicken genome expansion ratio

The large variation in genome size between organisms¹⁴³ has been addressed previously by comparing entire genomes, thereby deriving an aggregate estimate for expansion or contraction in one lineage. The change in overall genome size between chicken and mammals coupled with the conserved synteny allows us to isolate different factors that contribute to this variation (see Methods). The chicken genome is about 40% the length of the human genome, but this ratio is not constant across all orthologous regions (Fig. 15a). The variation in length ratios for human–chicken alignments is much greater than that seen for human–rodent alignments (Fig. 15b). The higher density of interspersed repeats in the human genome accounts for ~10% of this variation (correlation coefficient 0.326, $P < 10^{-4}$), with the strongest positive association at intermediate ranges (Fig. 15c). G+C content also accounts for a portion of the residual variability: after subtracting repeats from human and chicken in each window, the resulting length and G+C content ratios are strongly and negatively related (correlation coefficient -0.256 , $P < 10^{-4}$) (Fig. 15d).

More extensive multivariate regression analyses using these and other genomic parameters derived from human–chicken alignments explain only 20–25% of the variability in length ratios (see Methods and Supplementary Table S6). Features not measured in the current analysis are thus the main contributors to changes in genome size. One potential hidden variable is the density of ancient

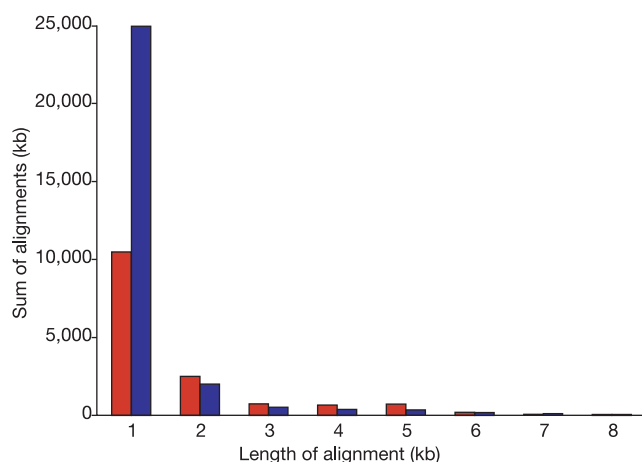


Figure 13 Segmental duplications within the chicken genome are small. The length distribution of intrachromosomal (blue) and interchromosomal (red) alignments that were confirmed by over-representation in the whole-genome shotgun sequence is shown for the chicken genome. The sum of alignments for alignments of lengths 9–15 kb are each less than 40 kb (data not shown).

Table 11 Comparison of orthologous segments

Feature	Anchors	
	Orthologous genomic segments	Orthologous gene pairs
Number of blocks	1,068	1,009
Average size in human (Mb)	2.10	2.03
Average size in chicken (Mb)	0.78	0.76
Average human:chicken ratio	2.67	2.67
Median size in human (kb)	177	566
Median size in chicken (kb)	74	185
Median human:chicken ratio	2.39	3.06
Longest block in human (Mb)	91.5	48.5
Longest block in chicken (Mb)	38.2	19.8
Coverage per chicken chromosome (%)	71.1	66.1
Coverage per human chromosome (%)	72.7	66.8

repeat sequences that have diverged beyond the ability to be found confidently by RepeatMasker¹⁴⁴. For example, repeat expansions in G+C-poor regions during early mammalian evolution might contribute to length ratio variation.

Illuminating the human genome: conserved non-coding segments

One important use of the chicken sequence is to identify functional non-coding elements in the human genome. The high synonymous substitution rates described above would preclude neutral regions from aligning between human and chicken. Thus the regions that do align are likely to be subject to purifying selection and hence are predicted to be functional. Compared with approximately 5% of the human sequence estimated to be under purifying selection, based on human–mouse comparisons^{2,145}, only 2.5% of the human sequence aligned with chicken. Of aligned positions, 44% are in protein-coding regions, 25% are intronic and 31% are intergenic. Aligned sequences within genes were discussed above in relation to chicken gene content. Here we investigate the global properties of the complete set of non-coding aligned segments.

Conservation patterns in proximal *cis*-regulatory regions

We examined functional non-coding elements in the human genome to analyse how frequently they are conserved with chicken. For sets of known regulatory elements, functional promoters, predicted promoters, CpG islands, and predicted transcription-factor-binding sites that are conserved between human and rodents, we find that only 30–40% are conserved between human and chicken (Fig. 16). Even for coding exons, which are among the most highly constrained elements in the genome, only 75% aligned between human and chicken. Thus, conservation between mammalian and chicken genomes greatly increases specificity in the search for functional elements, but at a price in sensitivity that varies depending on the functional category. The conserved regulatory modules

(CRMs; for example, promoters and enhancers) that still align between human and chicken are not a coincidental result of a lower local rate of neutral divergence because the distribution of neutral substitution values^{2,126} for the regions containing conserved CRMs was indistinguishable from that for the diverged CRMs. We assessed whether the biological functions of genes regulated by the conserved CRMs might differ from those with divergent CRMs. We found that genes from three GO categories (development, metabolism and structural component of muscle) are over-represented in the set regulated by the conserved CRMs, whereas genes within the signal transducer and hydrolase activity GO categories are over-represented in the set with non-conserved regulatory elements (Fig. 17).

Conserved non-coding regions are clustered and far from genes

Several recent human–rodent comparisons^{146–148} within limited regions of the genome have observed that conserved non-coding segments tend to lie relatively far from genes. To investigate whether this holds true across the genome in human–chicken comparisons, we partitioned the human genome into non-overlapping intervals, and in each interval (after excluding repetitive nucleotides) computed the fraction that falls in a coding exon annotated by RefSeq or Ensembl (the ‘coding fraction’) and the fraction that aligns with chicken but does not intersect coding exons (the ‘conserved non-coding fraction’ or CNF), taking care to keep the two quantities logically independent (see Methods). The two quantities are negatively associated (correlation coefficient -0.197 , $P = 0.000$; Supplementary Fig. S15). This inverse correlation is robust to variation in the interval size (between 100 and 1,000 kb), the set of gene annotations, and the set of non-coding alignments, and it is not explained by G+C content alone (see Methods). Proximal *cis*-regulatory elements (that is, those within a few kilobases of an exon) thus seem not to be the primary contributors to overall non-coding conservation.

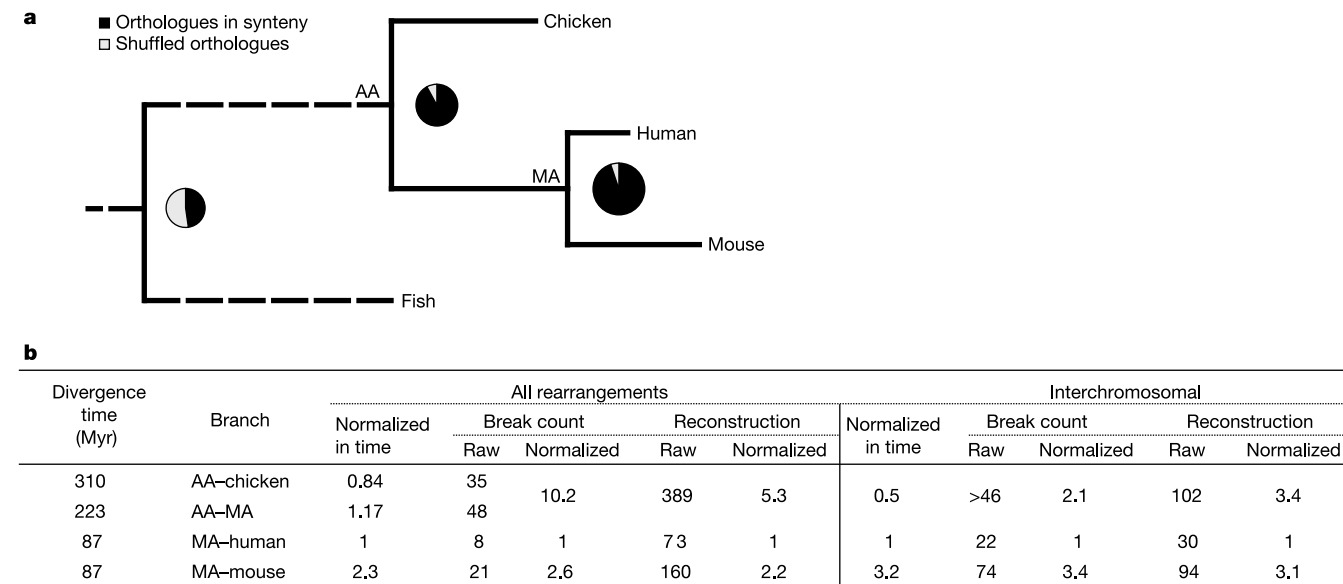


Figure 14 Rates of genome structure divergence. **a**, Phylogenetic tree of chicken, human and mouse showing rates of genome structure divergence, where the branch length is proportional to the number of estimated chromosomal rearrangements. Details are given in the supporting table **(b)**. AA, amniote ancestor; MA, mammalian ancestor. The pie charts show the fraction of orthologous genes that have retained their genomic neighbourhood; for example, about 85% of chicken and human orthologous genes reside in orthologous chromosomal segments, and their sizes are proportional to the number of recognizable orthologous genes. **b**, The table highlights a very low rate of interchromosomal shuffling in early mammalian and bird evolution, and an elevated rate

of interchromosomal shuffling in the rodent lineage. It provides a summary of the number of chromosomal rearrangements estimated by counting synteny breaks where ancestral state is supported by synteny to an outgroup species, and by reconstruction of ancestral genomes through a combinatorial search for the most parsimonious rearrangement pattern. The two methods agree reasonably and have been used to estimate the relative branch lengths of the genome structure divergence tree in **a**. Normalization to the length of the MA–human branch and to the time of independent evolution is presented for easy comparison.

Non-coding conservation is not uniformly distributed across the human genome. Fifty-seven segments of high non-coding conservation (average length 1.176 Mb and average CNF 13.1%, compared with a genome average of 1.7%; see Methods and Supplementary Table S7) were found to be gene poor (they cover 2.3% of the human genome but contain only 0.3% of the exons in RefSeq genes). They

were also G+C poor (38.8% overall) and depleted for all classes of interspersed repeats, as are their chicken orthologues. They contain none of the 731 break points identified through an analysis of regions of conserved gene order between human and chicken with length exceeding 200 kb, and—from human–mouse neutral substitution rates estimated using interspersed repeats¹²⁶—do not seem to have experienced particularly low mutation rates. The genes within or overlapping these 57 high-CNF segments, however, are significantly enriched for the GO categories associated with gene regulation (Fig. 17). Thus, the degree of non-coding sequence conservation is related to the biological function of the genes in the general genome neighbourhood. This enrichment is not merely a by-product of the gene-poor nature of these segments, because human gene-poor regions with low CNF are not enriched for the same GO categories¹⁴⁹.

The 57 high-CNF segments were found to contain 60.7% of 417 human–chicken ultraconserved elements (UCEs): sequences defined as being 200 bp or longer in orthologous chromosomal locations and 100% identical between the two species. Only 27.3% of these human–chicken UCEs overlap the 481 previously studied human–rodent UCEs¹⁵⁰, although all of them are found in the draft genomes of either mouse or rat, at 87–100% identity in the more conserved rodent. Human–chicken UCEs differ from human–rodent UCEs in containing far fewer exon-intersecting elements (7.9% versus 23%) and in showing only weak enrichment for proximity to genes whose products have a GO and InterPro classification associated with RNA splicing. The two UCE sets share a strong bias against harbouring human-verified single-nucleotide polymorphisms ($P < 10^{-40}$ compared to the genome-wide average in both cases). In both, the set of elements with no expression evidence (non-exons in ref. 150) is found in or next to genes whose products are highly enriched for transcriptional regulation, DNA binding, homeodomains and developmental functions; many of these UCEs are found more than 100 kb away from the corresponding genes. Finally, in both sets, no UCEs except a handful of coding regions could be traced back through sequence similarity to *Ciona intestinalis*, *Caenorhabditis elegans* or *Drosophila melanogaster*. At the moment, little is understood about the functional significance of either the UCEs or the high-CNF segments that are far from genes.

Conclusion

The chicken genome represents an intermediate test case as a target

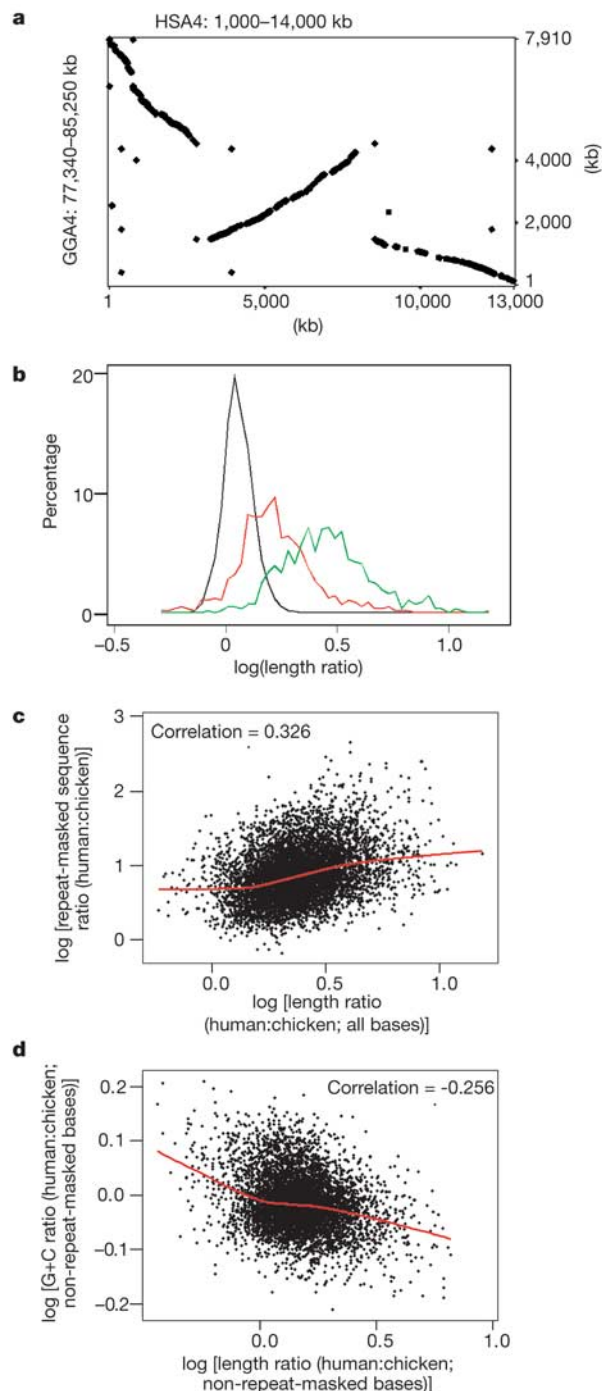


Figure 15 Variation in the ratio of lengths of human and chicken DNA in aligned segments. **a**, Dot plot comparing orthologous regions of human and chicken, showing variable slope. **b**, Variation in log-transformed ratio of lengths of aligned segments, comparing human–chicken (green, all; non-repetitive, red) and human–mouse (black). **c**, Scatter plot of the log-transformed ratio of lengths of orthologous human and chicken segments versus ratio of repeat-masked sequence in the two species. **d**, Scatter plot of the log-transformed ratio of lengths versus ratio of G+C contents after removal of masked bases. Lowess smooths (locally weighted scatterplot smoothing) are superimposed (red curves; smoothing parameter 0.5). See Methods for details.

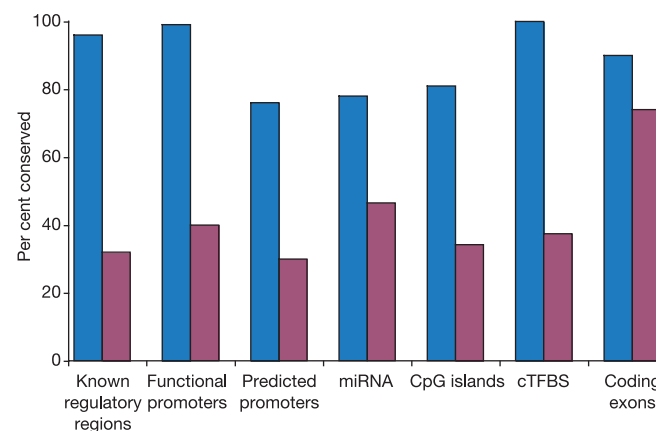


Figure 16 All sets of functional elements in human–chicken alignments show reduced representation relative to human–mouse–rat alignments. We examined functional elements containing known regulatory regions^{2,164}, functional promoters and predicted promoters¹⁶⁵, miRNA³⁴, CpG islands, conserved transcription factor binding sites³, and coding exons of known genes. The per cent of each category that aligns is shown for the human–mouse–rat alignments (HMR, blue) or human–chicken alignments (HC, red).

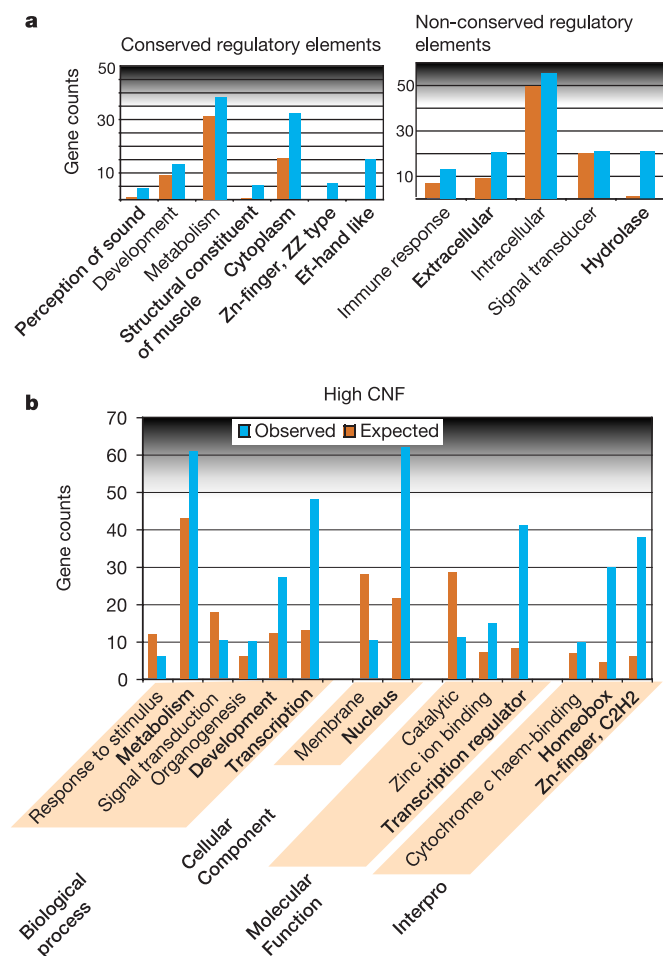


Figure 17 Enrichment of particular GO categories in genes regulated by conserved or non-conserved *cis*-regulatory modules (a) and in high-CNF regions (b). The observed and expected gene counts are shown for GO categories with at least five genes from high-CNF segments. Significantly enriched categories are in bold; the largest significant *P*-value is 0.0022 and the others range from 10^{-10} to 10^{-25} .

for genome sequence assembly and analysis. Although less than half the size of mammalian genomes, it is still much larger than those of *D. melanogaster*, *C. elegans* and even *T. rubripes*, and it lacks the dense linkage map platform that helped to assemble the first two. Unlike the rat and chimpanzee genomes, there was no closely related, high-quality genome sequence already available to provide a framework for assembly. Nevertheless, a relatively high-quality draft of the chicken genome has been achieved on the basis of only $6.6 \times$ whole-genome shotgun coverage, owing in part to the remarkably low level of recent transposon activity it has endured (Table 5).

The quality of this draft genome sequence makes it a key resource for comparative genomics. Natural selection and evolution provide us with many perspectives from which to view our own genome. Genomes of distant species resolve key processes that have been conserved over millennia, whereas those of our close relatives allow an analysis of rapidly changing sequence. In many respects, the chicken genome provides insights that were unavailable from previous sequences. For nearly every aspect of biology, it allows us to distinguish features of mammalian biology that are derived or ancient, and it reveals examples of mammalian innovation and adaptation.

The chicken is sufficiently distant that little unselected sequence has survived unchanged along the separate evolutionary paths to birds and mammals from their last common ancestor. Against this

background, conserved non-coding sequences stand out clearly. Some of these represent known regulators (Fig. 16) and others use novel mechanisms yet to be identified. On the other hand, the counterparts of many functional mammalian sequence elements could not be identified in the chicken sequence. Either these represent mammalian innovations or else any commonality has been lost over the course of >310 Myr of separate mutation and fixation.

Chicken breeding, based on quantitative genetic methods, represents one of the most remarkable examples of directed evolution. Even after 50 yr of intensive selection, annual genetic progress in production traits remains undiminished¹⁵¹. An impressive list of chicken quantitative trait loci has already been identified¹⁵², many with combined agricultural and medical relevance. The chicken genome sequence promotes both the development of more refined polymorphic maps (see the accompanying paper¹⁵³) and the framework for discovering the functional polymorphisms underlying interesting quantitative traits, thus fully exploiting the genetic potential of the chicken.

The chicken genome is invaluable for shedding light on functional elements of the human genome and our unique evolutionary history. It also points the way forward to the great utility we can expect from the genome sequences of other carefully chosen species. The data presented here demonstrate both the unique value of the chicken as a model species and emphasize the incomplete nature of our collective understanding of complex organisms. This chicken genome sequence will both integrate and stimulate the expanding array of contributions from this versatile species. □

Methods

Domain matching and ranking

To identify known families of genes and domains we scanned respective proteomes for characteristic HMM profile signatures from Pfam⁶⁹ and SMART databases using HMMER (<http://hmmer.wustl.edu/>) software and applying corresponding family-specific cutoffs. The identified families were ranked by the number of matching genes requiring at least one matching transcript, and only counting repetitive matches once.

Orthology detection

Orthologous relationships between genes of chicken, human, *Fugu* and others were inferred through systematic similarity searches at the level of the predicted proteins. We retained only the largest predicted ORF per locus, and compared those in an all-against-all fashion using the Smith–Waterman algorithm. We then formed orthologous groups using a variant of a strategy used earlier^{137,154,155}. First, we grouped recently duplicated sequences within genomes into ‘paralogous groups’, to be treated as single sequences subsequently. For this, there was no fixed cutoff in similarity, but instead we started with a stringent similarity cutoff and relaxed it on each successive step, until all paralogous proteins were joined, thereby satisfying the following criteria: all members of a group had to be more similar to each other than to any other protein in any other genome; and all members of the group had to have hits that overlapped by at least 20 residues, to avoid ‘domain walking’. After grouping paralogous proteins, we started to assign orthology between proteins by joining triangles of reciprocal best hits involving three different species (here, paralogous groups were represented by their best-matching member). Again, a stringent similarity cutoff was used first and relaxed on each successive step, and all proteins in a group were required to have hits overlapping by at least 20 residues. Finally, we joined any remaining nodes by allowing not only reciprocal triangles, but also reciprocal tuples.

Detection of gene loss in mammals

The orthologous relations defined above were used to infer losses when a gene was found in chicken and in at least one earlier-branching animal, but not in any mammal. Of 122 candidate losses obtained in this manner, many were manually discounted after Blastp searches in mammalian genomes (thus hinting that several as-yet-unannotated genes in mammals remain to be predicted).

Detection of orthologous introns

For each orthologous group we created a multiple alignment and mapped intron positions and protein features onto it. This procedure is incorporated into the SMART web server. To minimize errors due to erroneous alignments, introns flanking alignment gaps were discarded (less than 1% of all introns). To compensate for effects of intron sliding and to reduce further the impact of possible alignment errors, we allowed a window of 12 nucleotides in which we considered a position as conserved. Previous estimates indicated that the chance of independent intron insertion in such a window is $<1\%$. To avoid biases due to incomplete gene predictions, we omitted 18,910 introns in regions that were missing from some of the predicted genes.

Deriving tissue expression data

Chicken ESTs were mapped to the assembly, and to Ensembl genes (± 1 kb), using BLAT and a 95% identity threshold, and were partitioned into ten (brain; fat and skin; bone and connective tissues; heart; kidney and adrenal tissues; immune; liver; female reproduction; alimentary; testis) distinct tissue types. Percentage amino acid sequence identities of 1:1 chicken–human orthologues were calculated as described previously (Fig. 6). Note that single genes may be assigned to multiple tissues.

Whole-genome alignments

Human–chicken whole-genome alignments were obtained by using the program BLASTZ¹⁵⁶ to produce short (typically 100–1,000 bp) local alignments, and then assembling gap-free segments of those alignments into ‘chains’ in which aligned segments occur in the same order and orientation in both species¹³⁴. These alignments—which were used to generate data for Figs 2, 15–17 and Table 4, and Supplementary Figs S1, S15 and Supplementary Tables S6 and S7—can be obtained from the U.C. Santa Cruz Browser (<http://genome.ucsc.edu/>). To compute the CNF of a human genomic interval, we limited consideration to non-repetitive bases that are not in a local alignment that intersects a protein-coding region, and determined the fraction of those bases that are within an alignment.

Evolution of vertebrate genomes

The maps of conserved synteny (orthologous chromosomal segments with a conserved gene neighbourhood^{133,134,137}) between chicken, human and mouse were produced using whole-genome DNA alignments post-processed into chains and nets¹³⁴ as well as looking for a conserved neighbourhood of orthologous genes as described previously^{133,137}. We used gene-based synteny as input to MGR¹⁵⁷ and GRIMM^{133,158} to look for parsimonious scenarios of rearrangements, starting from a set of 6,447 four-way orthologous genes pre-filtered for evidence of conserved pairwise synteny using SyntQL (E. Zdobnov, unpublished program) and applying GRIMM–Synteny¹³³ for more stringently defined 586 four-way human–mouse–rat–chicken synteny blocks.

Detailed descriptions of all methods are provided in the Supplementary Information.

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A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms

International Chicken Polymorphism Map Consortium*

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We describe a genetic variation map for the chicken genome containing 2.8 million single-nucleotide polymorphisms (SNPs). This map is based on a comparison of the sequences of three domestic chicken breeds (a broiler, a layer and a Chinese silkie) with that of their wild ancestor, red jungle fowl. Subsequent experiments indicate that at least 90% of the variant sites are true SNPs, and at least 70% are common SNPs that segregate in many domestic breeds. Mean nucleotide diversity is about five SNPs per kilobase for almost every possible comparison between red jungle fowl and domestic lines, between two different domestic lines, and within domestic lines—in contrast to the notion that domestic animals are highly inbred relative to their wild ancestors. In fact, most of the SNPs originated before domestication, and there is little evidence of selective sweeps for adaptive alleles on length scales greater than 100 kilobases.

The generation of a high-quality draft sequence of the chicken genome (*Gallus gallus*) is an important advance in the field of animal genetics¹. Chickens are good models for studying the genetic basis of phenotypic traits because of the extensive diversity among domestic chickens that have been selected for different purposes. Monogenic traits are well studied^{2–4}, but many interesting traits are complex and determined by an unknown number of genes. Quantitative trait loci (QTLs) have been mapped for a range of traits, including ones for growth, body composition, egg production, antibody response, disease resistance and behaviour⁵. Determining causative genes for quantitative traits is difficult because each locus controls only a fraction of the phenotypic variance. We describe a survey of the genetic variation between three domestic chicken breeds and their wild ancestor. The 2.8 million SNPs that we identified will facilitate mapping of complex traits in many ways. First, improved marker density allows researchers to take advantage of the higher recombination rates in chicken¹, which are 2.5–21 cM Mb^{–1} depending on the chromosome, as compared with ~1 cM Mb^{–1} for human and ~0.5 cM Mb^{–1} for mouse. The previous linkage map used 2,000 markers^{6,7}, but only 800 of those were microsatellites or SNPs, which are the most useful⁸. Our new data allow researchers to construct detailed haplotypes that segregate in different QTL crosses. Because any mutation underlying a QTL must once have originated from a single founder animal, haplotype comparisons will facilitate the fine mapping of QTLs⁹. To this end, we conduct a genome-wide search for evidence of selection due to domestication, and provide an initial characterization of the expected magnitude of these effects.

Genetic variation and utility

Our experiment is outlined in Fig. 1. SNPs are generated by partial sequencing at one-quarter coverage for each of three domestic breeds (a male broiler, a female layer and a female silkie). The resultant reads are then compared with the genome (at 6.6× coverage) for the wild ancestor of domestic chickens, red jungle fowl. We expect marked heterozygosity within the three domestic lines, but not within red jungle fowl, because the bird that was sequenced for the genome project came from a highly inbred line that is essentially homozygous.

Comparison of the sequence reads for broiler, layer and silkie to the genome of red jungle fowl revealed nearly one million SNPs in

each instance, at mean rates of about five SNPs per kilobase (kb) (Table 1). Note that all of the SNP rates stated in this paper are calculated as nucleotide diversities (π), and given in units of $\pi \times 10^3$. After correcting for SNPs detected in more than one line, there are 2,833,578 variant sites or one potential marker for every 374 base pairs (bp) along the 1.06 gigabase (Gb) genome. To assess the reliability of these data, we resequenced 295 SNPs in the same bird in which they were detected (Supplementary Table S1). As much as 94% of the SNPs were confirmed; however, confirmation rates are sensitive to functional context (for example, coding versus non-coding) and SNPs in rare categories are less likely to be confirmed. For example, only 83% of the non-synonymous SNPs were confirmed. Small indels (insertions or deletions) of a few base pairs in length (mean of 2.3 bp and median of 1 bp) are detectable at rates that are well correlated with the corresponding SNP rates, but smaller by about a factor of ten.

Chicken autosomes are sorted by size into five large macrochromosomes (*G. gallus* (GGA)1–5), five intermediate chromosomes

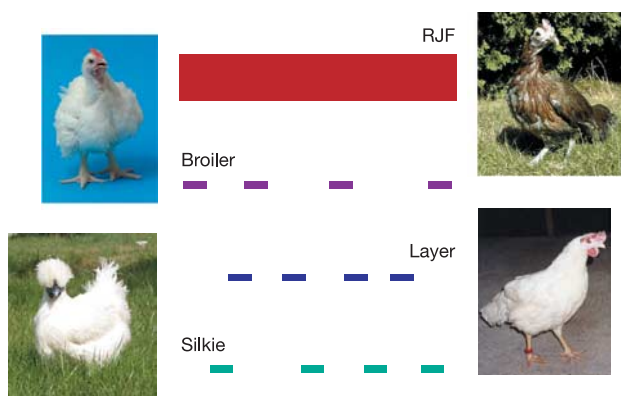


Figure 1 SNP discovery experiment. We sampled three domestic chickens at one-quarter coverage each and compared the resultant sequence to the 6.6× draft genome of red jungle fowl (RJF). Chicken photographs are provided by B. Payne (red jungle fowl), P. M. Hocking (broiler), L. Andersson (layer) and N. Yang (silkie).

Table 1 Frequency of SNPs in comparisons of red jungle fowl and three domestic chicken lines

Comparison	Number of SNPs	<i>L</i> (effective)	SNPs kb ⁻¹
Wild versus domestic			
RJF–broiler	1,041,948	197,431,517	5.28
RJF–layer	889,377	170,586,544	5.21
RJF–silkie	1,217,817	217,841,171	5.59
Between domestic lines			
Broiler–layer	194,605	37,506,800	5.19
Broiler–silkie	257,849	47,554,311	5.42
Layer–silkie	246,954	42,682,304	5.79
Within domestic lines			
Broiler–broiler	59,227	13,835,075	4.28
Layer–layer	40,412	10,863,595	3.72
Silkie–silkie	83,630	15,253,383	5.48
Compare with layer BACs			
RJF–BAC	20,925	3,809,567	5.49
BAC–broiler	4,404	847,456	5.20
BAC–layer	3,904	740,392	5.27
BAC–silkie	5,089	925,738	5.50

Comparisons involving 3.8 Mb of finished BAC sequence from another line of the layer (white leghorn) breed is shown at the bottom. SNP rates are an estimate of nucleotide diversity (π), as embodied by the effective length (*L* (effective); in bp), which considers how much of the data are of sufficiently good quality to actually detect SNPs and the probability that overlapping reads might be derived from homologous chromosomes. RJF, red jungle fowl.

(GGA6–10) and 28 microchromosomes (GGA11–38). SNP and indel rates are independent of chromosome size, as shown in Fig. 2. GGA16 is the only exception, because it contains the highly variable major histocompatibility complex (MHC). (There are only 20 kb of aligned sequence on GGA16, and if we were to remove it, the total SNP rate would only change by 0.02%.) This result is surprising because recombination rates on microchromosomes are much higher than on macrochromosomes¹, and studies in other organisms reveal a positive correlation between recombination rates and polymorphism rates^{10,11}. We suspect that higher gene densities on microchromosomes counteract the effect of higher recombination rates.

SNP rates between and within chicken lines can be determined from the overlaps between reads. Table 1 demonstrates that almost every pairwise combination gives a SNP rate of just over 5 SNPs kb⁻¹, except for broiler–broiler and layer–layer, which show about 4 SNPs kb⁻¹ (as expected because the sequenced broiler and layer are from closed breeding lines). To ensure that there are no confounding factors from the single read nature of our data or the complexities of the overlap analysis, we used comparisons to 3.8 Mb of finished bacterial artificial chromosome (BAC) sequence of a different white leghorn¹²—from the same breed but not the same

Table 2 Frequency of sequence polymorphisms between red jungle fowl and broiler

Gene set	SNPs kb ⁻¹	Indels kb ⁻¹	Number of SNPs	Number of indels
Confirmed mRNA transcripts				
5' UTR	3.45	0.46	203	27
Coding region	2.11	0.05	1,772	41
<i>K_A</i>	0.73	—	—	—
<i>K_S</i>	7.44	—	—	—
Introns	5.70	0.52	86,586	7,915
3' UTR	3.40	0.42	1,946	243
Human disease genes				
Coding region	2.74	0.04	1,005	15
<i>K_A</i>	1.10	—	—	—
<i>K_S</i>	9.40	—	—	—
Introns	5.36	0.49	27,768	2,553
Ensembl (final version 040427)				
5' UTR	4.22	0.37	616	54
Coding region	2.71	0.06	12,229	276
<i>K_A</i>	1.17	—	—	—
<i>K_S</i>	8.28	—	—	—
Introns	5.64	0.52	367,361	33,869
3' UTR	3.92	0.43	2,130	236
Human–chicken motifs	2.41	0.25	3,636	379
Genome-wide average	5.28	0.48	1,041,948	94,578

Data are sorted by functional context based on three non-redundant gene sets of 3,868 confirmed mRNA transcripts, 995 chicken orthologues of known human disease genes, and 17,709 Ensembl annotations. Human–chicken motifs are conserved sequences that exhibit no evidence of being genic in origin. Gene regions are subdivided into 5' UTR, coding exon, intron, and 3' UTR. *K_A* and *K_S* indicate non-synonymous and synonymous rates per available site.

line as the layer sequenced herein. Fifteen chromosomes were sampled and the results confirm our rates of 5 SNPs kb⁻¹. In another study of 15 kb of introns in 25 birds from ten divergent breeds of domestic chickens¹³, an autosomal rate of 6.5 SNPs kb⁻¹ was reported.

To quantify SNP and indel rate variation versus functional context, we considered three gene sets representing 3,868 confirmed messenger RNA transcripts, 995 chicken orthologues of human disease genes, and 17,709 Ensembl annotations from the red jungle fowl analysis¹. Complete details for all three lines are tabulated in the Supplementary Information (Supplementary Table S2). An excerpt for broiler is shown in Table 2. Within genes defined by mRNA transcripts, the SNP rates are 3.5, 2.1, 5.7 and 3.4 SNPs kb⁻¹ in 5'-untranslated region (UTR), coding exon, intron and 3'-UTR regions respectively. In coding regions, indel rates are 43 times smaller than SNP rates. The *K_A/K_S* ratio (where *K_A* and *K_S* are the number of non-synonymous and synonymous substitutions per available site) is 0.098, similar to what is typically seen in vertebrate comparisons. We also studied 'conserved non-coding regions' from

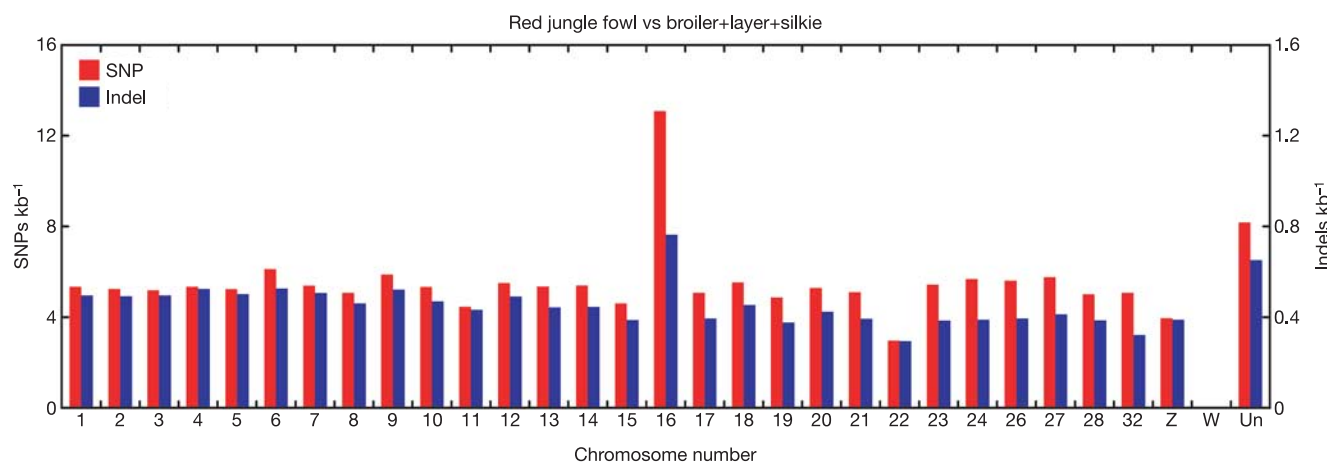


Figure 2 SNP and indel rates versus chromosome number. We excluded all sequences with 'random' chromosome positions. Because of the assembly problems on

chromosome W, it is not shown. The rates are computed as an average of all three domestic lines.

the red jungle fowl analysis¹. SNP rates are similar to those for coding exons, but indel rates are intermediate to those for coding exons and UTRs, thereby supporting the notion that these regions are functional but may not encode proteins.

The utility of these SNPs depends on their frequency of occurrence in commonly used chicken populations. Hence, we analysed 125 SNPs (including coding and non-coding SNPs, randomly distributed across the chicken genome) in ten unrelated individuals from each of nine divergent lines representing an assortment of European breeds. This collection includes commercial broiler and layer breeds, standardized breeds selected for their morphological traits, and an unselected breed from Iceland (Supplementary Table S3). Both alleles segregated in 73% of 1,113 successful marker–line combinations (out of 1,125 possible combinations). Averaged minor allele frequency is 27%, but it decreases to 20% if marker–line combinations where one of the two alleles is fixed are included. This indicates that most of the SNPs are common variants that predate the divergence of modern breeds. Only 12% of the markers had a minor allele frequency of less than 10% in the 90 animals tested.

We now demonstrate by example how these data can be used to target specific genome regions. Details of our experiments are in the Supplementary Information. First, we consider a body-weight-related QTL on GGA4 that was previously mapped to a 150-cM interval^{14,15}. After a year of effort, where every known microsatellite (>50) was tested, 26 informative markers were developed. Further progress would have required the laborious sequencing of multiple chickens to find additional polymorphisms in this target region. With the SNP map, we selected 47 random broiler–layer SNPs, and ABI SNPlex assays were developed to genotype an experimental F₂ cross ($n = 466$). Twenty-eight (60%) of these SNPs segregated in the cross but none showed breed-specific alleles, confirming that most variations predate domestication. In just one month we doubled the number of markers and resolved the initial QTL into two QTLs that affect body weight at 3 and 9 weeks of age.

In addition to providing markers for fine mapping, these SNPs are a rich source of candidate polymorphisms for the causative differences underlying important traits. As an example, candidate genes for disease resistance often include TGF- β ^{16,17}, cytokines¹⁸ and the MHC. We thus identified 40 SNPs from the SNP map in the coding or promoter regions of 12 cytokine genes. When analysed in eight inbred layer lines, 32 of these SNPs were informative. Cytokine genes on GGA13, including *IL4* and *IL13* (two genes that are expressed in T-helper-2 (Th2) cells), drive antibody response.

Four of the six SNPs that were polymorphic among lines were in *IL4* and *IL13*, and these SNPs were fixed for different alleles in lines N and 15I, which show differential antibody response to vaccination¹⁹. These SNPs therefore allow us to test whether the *IL4* and *IL13* loci directly determine the observed differential antibody response.

Domestication and selection

Domestic animals are useful models of phenotypic evolution under selection. The challenge is to find not only those loci that determine phenotypic differences, but also the causative alleles. We used two different approaches: first, searching for evidence of selective sweeps²⁰, and second, searching for non-synonymous amino acid substitutions at highly conserved sites. Given the available data, determining the exact haplotype structure is difficult because blocks of shared alleles can be erroneously disrupted by heterozygosity of the domestic lines and by sequencing errors. However, we can still search for the local reductions in heterozygosity that accompany selective sweeps, as long as we are mindful of the sequencing error rate. One example of a selective sweep is the *IGF2* locus in pigs²¹.

We carried out three-way comparisons of red jungle fowl and all possible combinations of two domestic lines. Given the limited coverage of the latter, we only examined 100-kb segments with at least ten SNP sites, where each qualifying site must have read coverage from every line. In practice, these segments contained an average of 25–28 SNPs. Then, we computed how often 80% or more of the SNP sites are identical in the two domestic lines but different in red jungle fowl. In Supplementary Table S4 we show that 0.4–1.5% of the segments qualified; however, when we searched for shared alleles between red jungle fowl and one domestic line, 1.2–2.6% of the segments qualified. We note that heterozygosity of the domestic lines is more of a confounding factor in searching for blocks of shared alleles between two domestic lines than between red jungle fowl and one domestic line. This could explain the difference, but if so, then heterozygosity of the domestic lines is the dominant factor in this analysis, not selective sweeps. Hence, selective sweeps that occurred before the divergence of modern domestic breeds must have left behind footprints that are much smaller than 100 kb. This would be consistent with the historically large effective population size of domestic chickens, and the reported high recombination rates.

For a glimpse of the true haplotype patterns one can compare the aforementioned 3.8 Mb of finished BAC sequence from the second layer line (L2) to the genome of red jungle fowl. These results are overlaid alongside the primary SNP data set in Fig. 3. Short red-

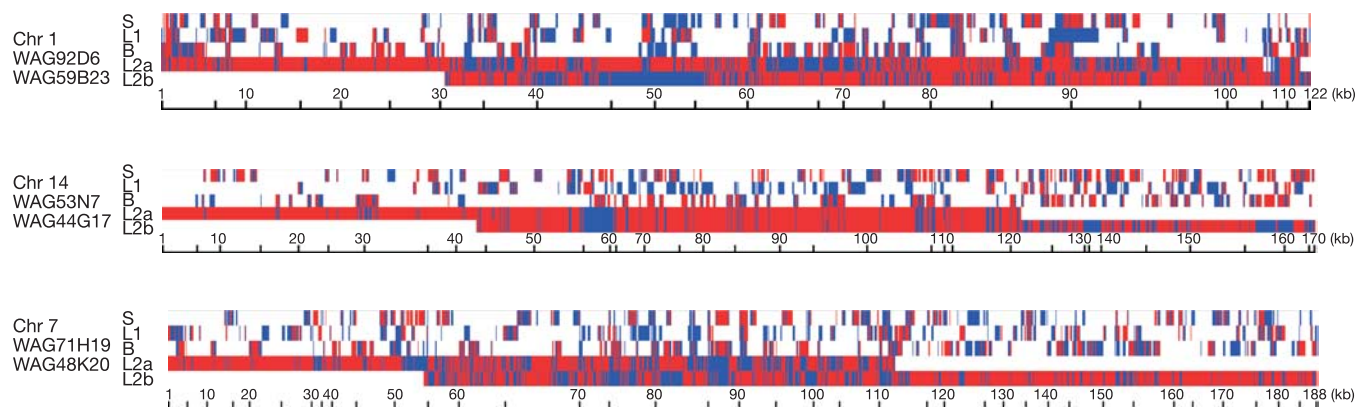


Figure 3 Detailed haplotype patterns in three regions, each covered by two overlapping BACs from the second layer line (L2). The primary SNP data are labelled B (broiler), L1 (layer) and S (silkie). All comparisons are to red jungle fowl, and we show only those sites where a SNP is identified in at least one of the four lines. Hence, the horizontal scale is

linear in the number of SNP sites, but nonlinear for size. Blue colours indicate where a particular line agrees with red jungle fowl, whereas red colours indicate where a particular line does not agree with red jungle fowl. Overlapping BACs on chromosomes 1 and 7, but not chromosome 14, are clearly from different haplotypes.

Ornithine transcarbamylase (OTC)	
188	
Human	HYSSLK G LTL S WIGDGN
Pig	--GA-----
Mouse	--G-----
Rat	--G-----
Chicken (WT)	--GG-N---IA-----
Chicken (Mut)	--GG-N R ---IA-----

Figure 4 Multi-species alignments for ornithine transcarbamylase (OTC), indicating non-synonymous substitutions relative to human protein. SIFT-intolerant position is indicated by site number and bold font. WT, wild type; Mut, mutant.

jungle-fowl-type fragments can be seen in all four lines. Shared domestic-type fragments can also be seen, but at sizes of 5–15 kb. This is consistent with our inability to detect footprints of selective sweeps at length scales of 100 kb, and suggests that a better choice of length is 10 kb. However, our data are insufficient for such a genome-wide analysis.

It has been proposed that loss-of-function mutations have accumulated in domestic animals as the result of relaxed purifying selection and selection for adaptive benefits²². An example of the latter is the deletion in the myostatin gene in cattle selected for muscularity²³. Such deletions are rare, and so we looked for non-synonymous SNPs at highly conserved sites using the program SIFT²⁴. Every substitution is thus classified as being likely to affect function (intolerant) or not (tolerant). For genes defined by mRNA transcripts, 26% of testable SNPs are intolerant, although only 11% are intolerant if we restrict this to high-confidence assessments (Supplementary Table S5). Usually, it is the domestic allele that is intolerant, but we would emphasize that intolerant SNPs are rare, and only 59% were confirmed by polymerase chain reaction (PCR) re-sequencing. Given that the domestic allele is represented by a single read, as opposed to 6.6 for the wild allele, much of this effect is probably due to sequencing errors. However, we noticed the same effect in 424 non-synonymous SNPs that we identified from an analysis of 330,000 expressed sequence tags (ESTs), where every allele was seen in two or more ESTs. We conclude that the loss-of-function hypothesis remains intriguing, but any effect is likely to be small.

Some of the experimentally confirmed SIFT-intolerant SNPs might be functionally important. We show one example in Fig. 4, from the ornithine transcarbamylase (OTC) gene. The SNP substitutes glycine in red jungle fowl to arginine in layer and broiler breeds. This SNP is identical to the G188R substitution associated with hyperammonaemia in humans²⁵. Re-sequencing of additional domestic birds revealed a high frequency for the intolerant allele in both white leghorns ($P = 0.65$, $n = 20$) and in broilers ($P = 0.75$, $n = 6$). In mammals, OTC is expressed in the liver and catalyses the second step of the urea cycle. Chicken OTC is expressed in the kidney and exhibits a low enzymatic activity, with substantial variability among breeds²⁶. Preservation and sequence conservation of OTC, along with all other enzymes in the urea cycle¹, was unexpected because avian species excrete uric acid (not urea) as their primary component of nitrogenous waste, and were believed to be lacking a functional urea cycle. The deleterious nature of human G188R makes this an attractive candidate for phenotypic studies of avian-specific adaptations in the urea cycle.

Discussion

This study provides the first global assessment of nucleotide diversity for a domestic animal in comparison to a representative of its wild ancestor. The small number of birds sequenced is compensated for by the vast number of sites examined. We detected surprisingly little difference in diversity in comparisons between red jungle fowl and domestic lines, between different domestic lines, and within domestic lines. The total rates are typically 5 SNPs kb⁻¹,

with the only exception being a slight reduction to 4 SNPs kb⁻¹ in broiler and layer lines that are maintained as closed breeding populations. Notice that our estimates do not include the female-specific W chromosome, which has a much lower genetic variability⁴⁸. In comparison, 5 SNPs kb⁻¹ is six- to sevenfold larger than humans²⁷ and domestic dogs²⁸, threefold larger than gorillas²⁹, but similar to the diversity between different mouse subspecies³⁰.

Most of the nucleotide diversity observed between and within domestic lines must have originated before the domestication of chickens 5,000 to 10,000 yr ago. Given a neutral substitution rate of 1.8×10^{-9} sites per year for galliform birds³¹, we estimate that a coalescence time of 1.4 million years would be required to account for the observed rates of 5 SNPs kb⁻¹. Considering that the rates observed between red jungle fowl and domestic lines are not much higher than those between domestic lines, it would seem that domestication has not resulted in a substantial genome-wide loss of diversity, as would be expected had a severe population bottleneck occurred. This is important because it contradicts the assertion that animal domestication began from a small number of individuals in a restricted geographical region³². That is still a possible scenario for the very earliest phases of domestication, but if so, our data imply that subsequent crossing with the wild ancestor (in the first 1,000 yr or so, until more developed breeds were established) restored this diversity. Nevertheless, extensive diversity is consistent with the ongoing improvements in agricultural traits that have been achieved over the last 80 yr in layer and broiler lines³³.

The most important application for this SNP map will be in analysis of QTLs and other genetic traits. Although the density of markers far exceeds what is needed for initial mapping, the principal challenge is not in the detection of linkage but in the identification of genes underlying QTLs⁹. By itself, our SNP map is not adequate. It must be combined with novel strategies and novel resources (such as mapping populations specifically designed for fine mapping). The essential problem is the lack of a one-to-one relationship between genotype and phenotype, as the latter is influenced by multiple genetic and environmental factors. This can be overcome, in experimental and domestic animals, by progeny testing and segregation analysis, which permit detailed characterization of haplotypes associated with different QTL alleles, and may eventually lead to the identification of the underlying causative mutations²¹. This SNP map will facilitate fine mapping.

As an example, the major Growth1 QTL on GGA1 explains about one-third of the difference between red jungle fowl and white leghorn in adult body weight and egg weight³⁴. Initial mapping assigned this locus to a ~20-cM confidence interval. Selective backcrossing using sires that have recombinant chromosomes, and QTL analysis using subsequent intercross generations, are currently being used to refine the localization to a few centimorgan, expected to be less than ~1 Mb. This establishes a collection of chromosomes of known QTL status. Our SNP map can then be used for haplotype analysis, assuming that the white leghorns share a chromosomal segment—identical by descent—with the causative mutation. The small haplotype blocks detected in this study underscore the need for a larger number of SNPs to identify such identical-by-descent segments. Although these small blocks may require greater marker density and more recombinants to identify the causative haplotype, less effort will be required to resolve the actual QTL alleles once the haplotype is found. □

Methods

Animals sequenced

Our broiler and layer lines are from European breeds with marked differences in meat and egg production traits. This specialization started only during the first half of the twentieth century³⁵. The sequenced male white Cornish broiler is from a closed breeding population commonly used in the production of commercial meat-type hybrids (Aviagen); effective population size is about 800. The female white leghorn layer is from a closed line developed at the Swedish University of Agricultural Sciences³⁶; its effective population size has been 60–80 birds for the past 30 yr. The Chinese silkie is used in meat/egg production

and traditional Chinese medicine³⁷. Selection intensity has been low, and the sequenced female is from a large outbred population.

DNA was extracted from the erythrocytes of a single bird, sheared by sonication, and size fractionated on agarose gels. Fragments of 3 kb in size were ligated to *Sma*I-cut blunt-ended pUC18 plasmid vectors. Single colonies were grown overnight, and plasmids were extracted by an alkaline lysis protocol. Sequences were read from both ends of the insert with vector primers and Amersham MegaBACE 1000 capillary sequencers. Roughly one million reads were generated for each bird. For broiler, layer and silkie we got a total of 841,790, 841,555 and 870,556 successful reads, with Q20 lengths of 380,729,199 bp, 372,263,344 bp and 397,831,117 bp, respectively.

Polymorphism detection

To minimize sequencing errors we use the Phred quality, $Q^{38,39}$. This is related to the single-base error rate by the equation $-10 \times \log_{10}(Q)$. We use more stringent thresholds than normal⁴⁰, with $Q > 25$ for the variant site and $Q > 20$ in both flanking 5-bp regions. For an indel, the variant site in the shorter allele is given the quality of its two flanking bases. We originally found many artefactual deletions relative to red jungle fowl, which upon a closer examination of the sequence reads were due to doublet peaks that got called as singlet peaks. This is an unavoidable flaw of the base-caller software. Hence, we raised the indel thresholds to Q30 and Q25. We must still advise caution, and to that end, indels in simple repeats are flagged and none are counted in Tables 1 and 2 and Supplementary Tables.

Paralogous confusion is detected in the course of the genome-level BlastN search that determines where the read is supposed to go. Once this is known, the detailed alignments are done within CrossMatch⁴¹. Analysis of the red jungle fowl genome¹ shows that recent segmental duplications typically agree to 2%. When the best and second best BlastN hits were more than 2% apart, and the best hit was not to a known segmental duplication, the best hit was taken. When either rule was violated, clone-end pairs information was used to resolve the ambiguity. Every alignment had to incorporate 80% of the read. Mapped back to the red jungle fowl genome, the amount of usable data for broiler, layer and silkie covered 190,513,980 bp, 165,154,746 bp and 210,214,479 bp respectively.

Rate normalization

Polymorphism rates are normalized to the length of the sequence on which we can detect SNPs. To correct for heterozygosity within a line, we calculate nucleotide diversity using the approximation⁴² $\pi = K / \sum_{i=1}^{n-1} \frac{1}{i}$, where K is the number of variant sites found by sequencing n chromosomes in a region of length L . When comparing red jungle fowl to one of the three domestic lines, n can only be 2 or 3, and it is a stochastic variable, because there is a 50% chance that any two overlapping reads are from the same chromosome. When there are m overlapping reads, the denominator is $\frac{1}{2m+1} (1 + (2^m - 1)(1 + \frac{1}{2}))$. We then sum over all possible regions, with different L and m values for each region, to get what we call the 'effective length'. Similar considerations are used to compute SNP rates within a line, except that n is 1 or 2, and as a result, the denominator becomes $\frac{L}{2m+1} (2^m - 1)$.

We calculate gene context relative to five different data sets. The first three are based on experimentally derived genes and the last two are based on computer annotations. Riken1 is a data set of 1,758 full-length complementary DNAs taken from bursal B cells of a 2-week-old Prague CB inbred⁴³. The second data set, GenBank, refers to 1,178 chicken genes with 'complete CDS' designation, downloaded as version 2003-12-15. BBSRC is a set of 1,184 full-length cDNAs taken from a larger group of 18,034 cDNAs⁴⁴ using TBlastX mapping to the vertebrate Refseq and BlastX mapping to SWALL. Through merging all three data sets we have 3,868 non-redundant genes. For the detailed gene models, we carried out a genome-level search in BLAT⁴⁵ and used SIM4 (ref. 46) to calculate the exon-intron boundaries. The last two data sets contain 995 chicken orthologues of human disease genes and 17,709 non-redundant Ensembl genes.

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TRPA1 is a candidate for the mechanosensitive transduction channel of vertebrate hair cells

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Mechanical deflection of the sensory hair bundles of receptor cells in the inner ear causes ion channels located at the tips of the bundle to open, thereby initiating the perception of sound. Although some protein constituents of the transduction apparatus are known, the mechanically gated transduction channels have not been identified in higher vertebrates. Here, we investigate TRP (transient receptor potential) ion channels as candidates and find one, TRPA1 (also known as ANKTM1), that meets criteria for the transduction channel. The appearance of TRPA1 messenger RNA expression in hair cell epithelia coincides developmentally with the onset of mechanosensitivity. Antibodies to TRPA1 label hair bundles, especially at their tips, and tip labelling disappears when the transduction apparatus is chemically disrupted. Inhibition of TRPA1 protein expression in zebrafish and mouse inner ears inhibits receptor cell function, as assessed with electrical recording and with accumulation of a channel-permeant fluorescent dye. TRPA1 is probably a component of the transduction channel itself.

Positive deflection of the mechanosensitive stereocilia bundle of vertebrate hair cells opens ion channels in the tips of the stereocilia^{1,2}. The extraordinary speed of channel opening after bundle deflection suggested that these transduction channels are directly gated by mechanical force on the channel protein, and that such channels represent a novel group of ion channels that are mechanically gated^{3,4}. Since then, a variety of mechanically gated ion-channel proteins have been identified in other systems⁵; however, the hair-cell transduction channel has remained elusive. The difficulty stems in no small part from the relative scarcity of the channel protein in the inner ear: each cell has only a few hundred functional transduction channels, largely precluding biochemical analysis.

Physiology has provided some clues: the transduction channel is a non-selective cation channel with a high Ca²⁺ permeability and ~100 pS conductance. It is permeable to some small organic cations and, remarkably, to the fluorescent lipophilic dye FM1-43 (refs 6–8). Whereas these characteristics rule out candidate channels of the DEG/ENaC family, which may mediate touch sensation in nematodes⁹, they are typical of ion channels of the TRP superfamily^{8,10,11}. Moreover, TRP channels are found in a variety of sense organs, probably mediating pheromone transduction (TRPC2), sweet and bitter taste (TRPM5), warm and cool thermal sensation (TRPV1–4, TRPM8, TRPA1), insect vision (TRP, TRPL, TRPL-γ), insect touch (NOMPC), and insect hearing (Nanchung)^{12,13}. The TRPN (also known as NOMPC) channels in *Drosophila* bristle organs have the same speed and sensitivity as the hair-cell channel¹⁴, and its homologue in zebrafish participates in hair-cell function¹⁵. Yet mammalian genomes apparently lack TRPN1.

To find transduction channel candidates, we searched a variety of genomes and identified channels within the TRP superfamily. *In situ* hybridization in the mouse inner ear for all mouse TRPs revealed

significant expression of TRPA1, a channel activated by mustard and cinnamon oils and by cold^{16–18}. We found that TRPA1 mRNA appears in the inner ear coincident with the onset of mechanotransduction, that antibodies to TRPA1 co-localize with the transduction apparatus, and that disruption of TRPA1 in zebrafish and mouse inhibits hair-cell transduction. The TRPA1 protein may thus be a subunit of the transduction channel itself.

In situ hybridization

The mouse genome encodes 33 ion channels of the TRP superfamily (M. Gupta, H.L.R., A.D. and D.P.C., unpublished results). To identify candidates for the vertebrate hair-cell transduction channel, we constructed *in situ* hybridization probes for all 33, and looked for expression in neonatal mouse inner ear. Three different probes for the mouse TRPA1 ion channel (mmTRPA1) strongly labelled the organ of Corti of the cochlea, which contains the auditory hair cells (Fig. 1a, c). To mark hair cells we also used an *in situ* probe to Math1 (Fig. 1b, d), a transcription factor essential for hair-cell specification¹⁹. Cochlear hair cells were lightly labelled with the *in situ* probe for TRPA1 (Fig. 1c), whereas stronger label appeared in Hensen's cells, a type of supporting cell adjacent to hair cells. Hair cells of the mouse sacculle (Fig. 1e) and semicircular canals (not shown) were also lightly labelled with the TRPA1 probes. Although labelling was not very strong in hair cells, this might be expected if the transduction channel is present at a few hundred copies per cell. Probes for other TRP channels (except for TRPML3; see ref. 20) did not label hair cells above background levels.

Expression during development

If TRPA1 participates in hair-cell transduction, its mRNA should appear just before hair cells become mechanically sensitive. The first

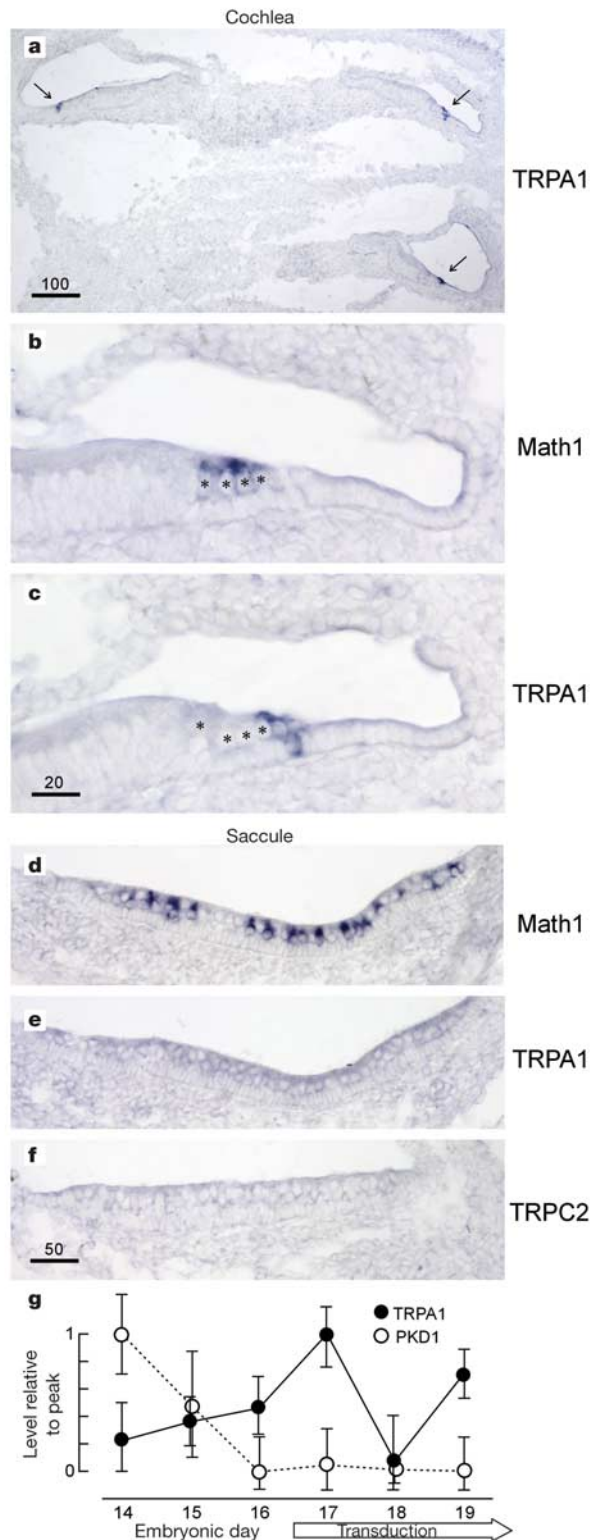


Figure 1 Expression of TRPA1 in the mouse inner ear. **a**, *In situ* hybridization at P0 for TRPA1 in cochlea (probe 194–1,020 bp). Arrows mark organ of Corti. **b**, *In situ* hybridization for Math1 in organ of Corti hair cells (asterisks). **c**, TRPA1, section adjacent to **b**. Hensen's cells are strongly labelled. **d**, Math1 in the mouse saccule. **e**, TRPA1, section adjacent to **d**. Hair cells are faintly labelled. **f**, TRPC2 as a negative control. **g**, Quantitative RT-PCR for TRPA1 and PKD1 during development. TRPA1 message rises just before the onset of transduction at E17. Data are mean $\pm$ s.d.; $N = 6$. Scale bars are in micrometres.

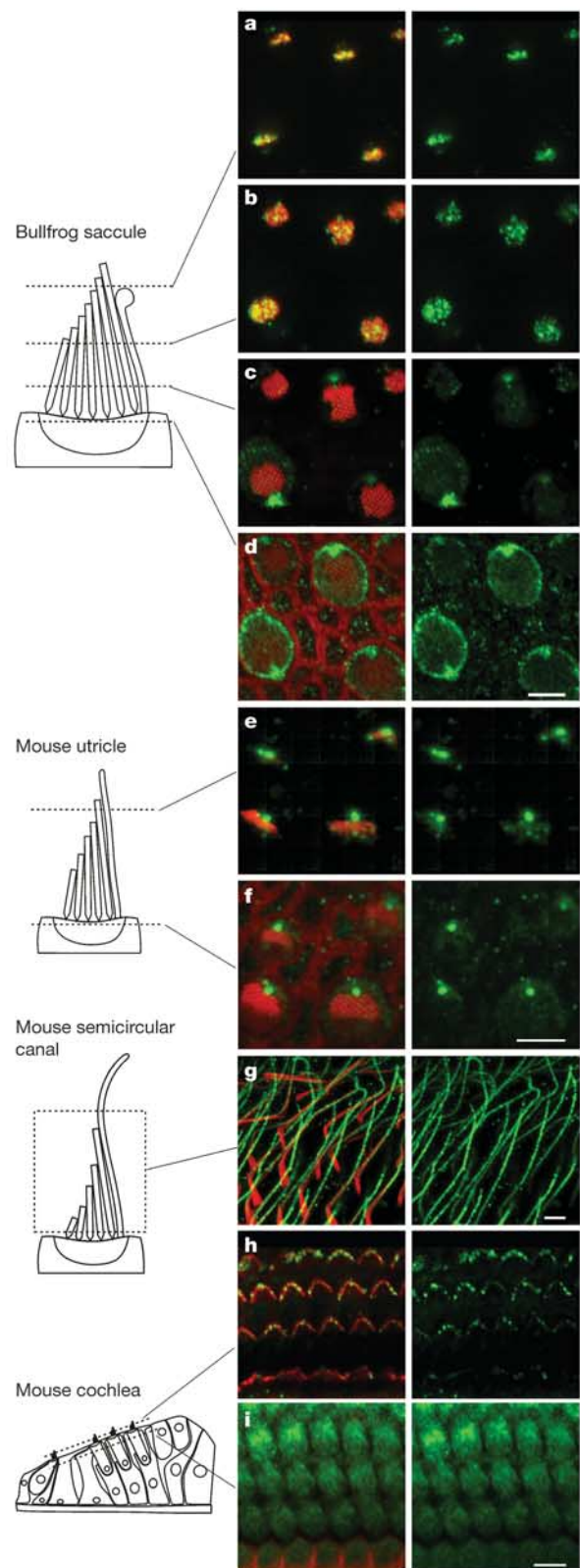


Figure 2 Antibody labelling of TRPA1 in bullfrog and mouse inner ears. Red, actin; green, TRPA1 antibody 0203mt. Right panel is TRPA1 antibody alone. **a–d**, Adult bullfrog saccule; optical sections as indicated. **e, f**, Hair cells from the mouse utricle; optical sections through a whole mount showing tips or bases of stereocilia. **g**, Hair bundles from the mouse semicircular canal. **h**, Hair cells from the mouse cochlea at P7. **i**, Label also appears in the cell bodies of hair cells. Scale bar, 5 μ m.

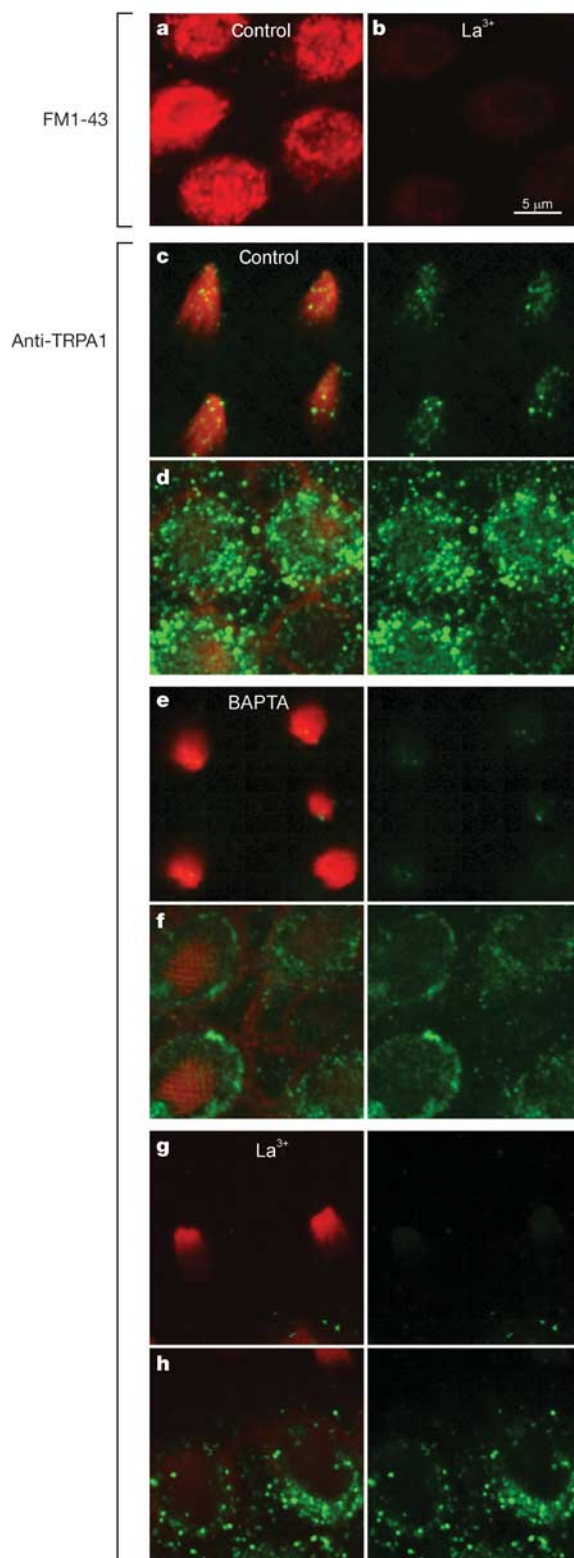


Figure 3 Redistribution of TRPA1 label upon disruption of the transduction apparatus. **a**, Accumulation of FM1-43 (red) in bullfrog hair cells during a 3-min exposure to the dye; optical section through the cell bodies. **b**, After treatment with 5 mM La^{3+} for 20 min. **c**, TRPA1 label (green) and actin label (red) in hair bundles of bullfrog hair cells. Average of two optical sections through the distal 3 μm of the bundles. **d**, Optical sections through the cuticular plate region showing the pericuticular zone. **e**, **f**, After treatment with 5 mM BAPTA for 20 min before fixation. **g**, **h**, After treatment with 5 mM La^{3+} for 20 min before fixation.

wave of mouse utricular hair cells, generated at embryonic day (E)12–13, acquires functional transduction at E17 (ref. 21). We used quantitative polymerase chain reaction with reverse transcription (RT–PCR) to assess message for several TRP channels during development of the mouse utricle (Fig. 1g). TRPA1 message was first detected at E15 and E16, and peaked at E17. Message level dropped at E18, but then rose again at E19, possibly corresponding to a second wave of hair cells that are generated at about E15 (ref. 22) and transduce by E19 (G.S.G.G. and J.R.H., unpublished data).

Antibody labelling

We raised antibodies to the carboxy terminus of mmTRPA1, after the sixth predicted transmembrane domain (Supplementary Fig. S1a). Antisera were affinity purified against either the entire C terminus (antibody 0203mt) or a small peptide representing conserved sequences within it (0203f1). In immunoblots, both the TRPA1 antibody (0203mt) and an antibody to green fluorescent protein (GFP) labelled a GFP::mmTRPA1 fusion protein expressed in HEK293 cells, indicating that our antibody recognizes the TRPA1 protein (Supplementary Fig. S2a).

Two other components of the transduction apparatus—myosin 1c (the adaptation motor) and cadherin 23 (a tip-link protein)—are located at the tips of the stereocilia, throughout the kinocilium and in the pericuticular zone (a vesicle-rich region of the apical surface)^{23,24}. To see whether TRPA1 was similarly located, we first looked in hair cells of the bullfrog saccule, whose large hair bundles facilitate antibody localization. TRPA1 immunoreactivity (antibody 0203mt) was found in the distal half of the stereocilia and along the length of the kinocilium (Fig. 2a, b). It was scarce or absent in the proximal part of the stereocilia (Fig. 2c), but strong in the pericuticular zone (Fig. 2d). Lighter labelling was also observed in the cell bodies of hair cells, but not supporting cells. We found a very similar labelling pattern with an alternatively purified antibody (0203f1; Supplementary Fig. S2g, h). Label was absent when the primary antibody was absent or when it had been pre-absorbed against the antigen (Supplementary Fig. S2e, f, i, j).

In hair cells of the mouse vestibular system stereocilia labelling was light, whereas the kinocilium and pericuticular zone were consistently labelled (Fig. 2e, f). The long kinocilia of semicircular canal hair cells showed robust label (Fig. 2g). In inner and outer hair cells of the mouse cochlea, immunoreactivity was strongest in the apical region of the hair cells but appeared lower in cell bodies as well (Fig. 2h, i). Labelling of the stereocilia tips was seen (Fig. 2h), especially in animals older than postnatal day 4 (P4), but was difficult to distinguish from cell body labelling in these short stereocilia. Kinocilia were consistently labelled at P3–P5. In Hensen's cells of the cochlea, where the *in situ* label was strongest, the Golgi apparatus was brightly labelled (Supplementary Fig. S4), further supporting the specificity of the antibody.

Because the tip link protein cadherin 23 is also present in retinal photoreceptors²⁵, we asked whether TRPA1 might be in photoreceptors. Indeed, in immunoblots of bullfrog retina, the TRPA1 antibody recognizes a single prominent band of the predicted molecular mass, and in sections TRPA1 immunoreactivity was found in the outer and inner segments of bullfrog photoreceptors (Supplementary Fig. S4).

In hair cells, tip links can be removed and mechanosensitivity abolished by a short treatment with La^{3+} ions or the Ca^{2+} chelator BAPTA^{26,24}. Notably, cadherin 23 immunoreactivity disappears from the stereocilia within 20 min after either treatment, suggesting that the transduction complex is rapidly recycled when damaged²⁴. If TRPA1 is part of the transduction complex, we might expect similar redistribution after disruption of the tip link. We first confirmed that La^{3+} and BAPTA abolish transduction channel function. Treatment of bullfrog saccular hair cells with either reagent prevented accumulation of the small fluorescent dye FM1-43, which passes through open hair-cell transduction channels

and is trapped in cytoplasm^{7,8}. La^{3+} was more effective, almost completely eliminating FM1-43 labelling in all hair cells, whereas hair cells at the edge of the sensory epithelium resisted BAPTA treatment (Fig. 3a, b).

We then treated bullfrog sacculi with either BAPTA or La^{3+} and followed TRPA1 redistribution with the 0203mt antibody. Both treatments caused substantial loss of TRPA1 immunoreactivity from the tips of stereocilia, reducing the levels of label by 75–85% (Fig. 3c–h). Immunoreactivity in the pericuticular zone was also reduced by La^{3+} (Fig. 3g, h), a pattern strikingly similar to that of the tip-link protein.

Morpholino oligonucleotides in zebrafish

We wondered whether inhibiting TRPA1 expression reduced transduction channel function. In zebrafish, protein expression can be

inhibited by injection of morpholino oligonucleotides (morpholinos) into early embryos. We first identified zebrafish homologues of TRPA1 from partial sequences in the *Danio rerio* genome. There are two, consistent with the duplication of the zebrafish genome, which we designate *trpa1* and *trpa2* (encoding drTRPA1 and drTRPA2, respectively). We determined the full-length coding sequences of both by PCR amplification from zebrafish complementary DNA and 5' and 3' rapid amplification of cloned ends (RACE) (Supplementary Fig. S1a, b). The drTRPAs are most closely related to each other and to a TRPA from the puffer fish, and are much more similar to channels of the TRPA branch than to any other TRP superfamily branch. To determine the exon structures of these genes, we compared their cDNA sequences to zebrafish genomic sequence (Supplementary Fig. S3, and data not shown).

Morpholinos expected to inhibit the translation or splicing of

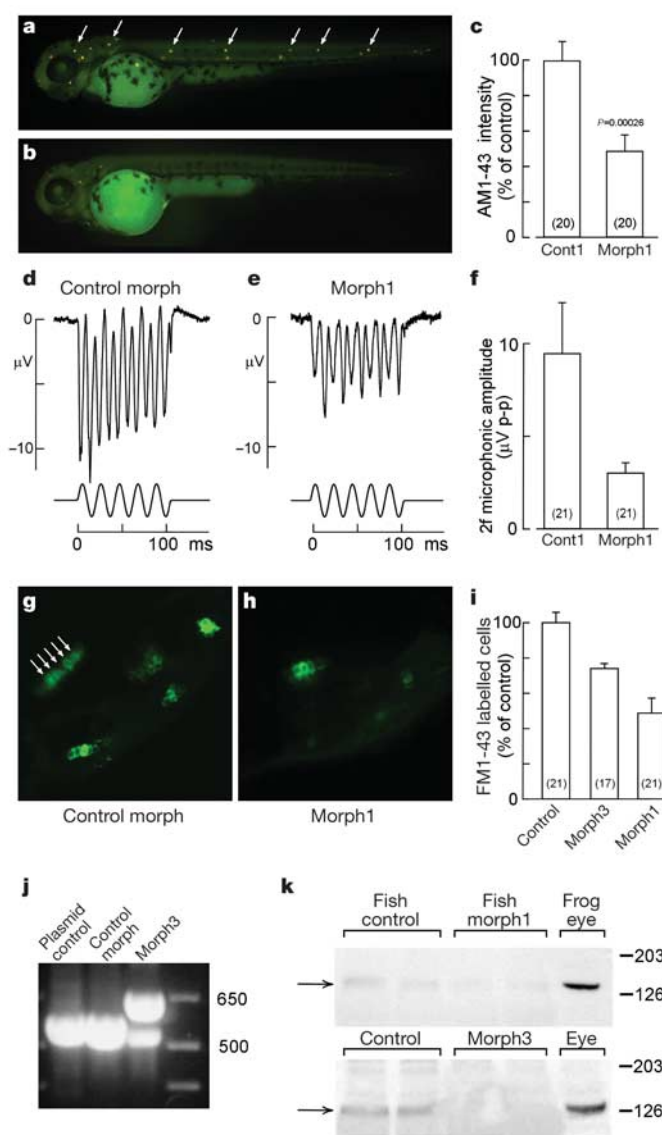


Figure 4 Inhibition of transduction in zebrafish by morpholinos. **a**, AM1-43 in lateral line hair cells (arrows); 60 h.p.f. embryo injected with control morpholino. **b**, Labelling of a 60 h.p.f. embryo injected with morph1. **c**, Total AM1-43 intensity in fish neuromasts. Data are mean ± s.e.m., $N = 20$, $P < 0.0003$. **d**, Microphonic potential in the otocyst of a control-injected fish. **e**, Fish injected with morph1. **f**, Summary of microphonic potentials; peak-to-peak (p-p) amplitude at twice the stimulus frequency (2f). Data are mean ± s.e.m., $N = 21$, $P < 0.03$. **g**, FM1-43 in zebrafish inner ear hair cells (arrows)

after dye injection into the otocyst. Projection of ~40 optical sections through a control-morpholino-injected zebrafish at 55 h.p.f. **h**, Otolocyst of a morph1-injected zebrafish. **i**, Summary of FM1-43-labelled hair cells. Data are mean ± s.e.m. **j**, RT-PCR of control plasmid and morpholino-injected embryos (60 h.p.f.). **k**, Immunoblot of whole 60 h.p.f. embryos injected with control morpholino, morph1 or morph3; bullfrog eye is positive control. Numbers along the right are bases in **j** and kDa in **k**.

mRNA for drTRPA1 and drTRPA2, as well as a standard control morpholino, were injected into zebrafish embryos at the one-cell stage. Hair cells of the zebrafish inner ear and lateral line become functional beginning at about 48 h post-fertilization (h.p.f.). After ~70 h.p.f., dilution and degradation of morpholinos apparently allow recovery from suppression and cause variability in morphant phenotypes¹⁵. We therefore tested hair-cell function between 58 and 72 h.p.f.

To assay lateral line hair cells, we bathed zebrafish embryos (60 h.p.f.) for 5 min in water containing FM1-43 or AM1-43 (a fixable analogue). Uninjected embryos and those injected with control morpholino showed brightly labelled hair cells (Fig. 4a). Fish injected with two different morpholinos targeting drTRPA2 were also labelled (data not shown), suggesting that drTRPA2 is not involved in hair-cell transduction. However, fish embryos injected with morpholinos targeting drTRPA1's translation initiation site (morph1) or a splice junction (morph3) showed neuromast fluor-

escence reduced to $44 \pm 9\%$ of controls (mean $\pm$ s.e.m., $N = 20$, $P < 0.0001$) or $74 \pm 3\%$ of controls ($N = 17$, $P < 0.0001$), respectively (Fig. 4b, c). DrTRPA1 is apparently necessary for the complete function or development of embryonic lateral line hair cells.

To assay inner ear hair cells of fish, we recorded the otocyst microphonic potential, an extracellular voltage resulting from current flow through transduction channels. Vibration of a stimulus pipette placed near the otocyst evoked a negative-going potential at twice the stimulus frequency (Fig. 4d), as expected if the stimulus alternately excited hair cells of opposite orientations. The potential became saturated for large amplitudes and its peak occurred slightly before the stimulus peak, as expected for hair cells; furthermore, it was abolished by the channel blocker streptomycin in the recording pipette. Fish embryos (58–70 h.p.f.) injected with a control morpholino consistently showed robust, stimulus-evoked microphonic potentials, whereas those injected with morph1 had potentials $32 \pm 5\%$ that of controls ($N = 21$, $P < 0.03$) (Fig. 4e, f).

Injection of morph1 and morph3 also reduced FM1-43 accumulation in inner ear hair cells. After physiological recording, we pressure-ejected a small amount of pipette solution, which contained FM1-43, into the otocyst. FM1-43 entered hair cells (but not other cells) within 2–3 min. Individual dye-labelled hair cells were counted in three-dimensional confocal reconstructions of each otocyst (Fig. 4g). About 30 hair cells were labelled with dye in control fish, but fewer were labelled in morph1- or morph3-injected fish ($49 \pm 8\%$ of control, $N = 21$, $P < 0.00001$ and $74 \pm 4\%$, $N = 17$, $P < 0.0001$, respectively). FM1-43 intensity in each cell also appeared lower than in controls.

To test whether the morpholinos had simply delayed hair-cell development, we fixed each dye-labelled embryo after confocal imaging. Permeabilization with acetone allowed FM1-43 to diffuse from hair cells. Hair bundles were labelled with fluorescent phalloidin, and the confocal reconstructions repeated. Control otocysts had 58 ± 2.5 hair cells with bundles, whereas morph1-injected fish had only slightly fewer (51 ± 2.5 , $N = 19$). Thus, the inhibitory effect of morph1 on transduction cannot be explained by fewer hair cells in the inner ear.

Finally, to confirm that the morpholinos did in fact disrupt drTRPA1 mRNA splicing and reduce protein levels, we extracted total RNA and protein from ~60 h.p.f. embryos injected with experimental and control morpholinos. By sequencing RT-PCR products, we found that morph3 caused a frameshift mutation and a stop codon insertion before drTRPA1's transmembrane domains by blocking splicing of intron 20–21 or by using a cryptic splicing donor site 22 base pairs (bp) 5' to the normal donor site (Fig. 4j). In immunoblots with antibody 0203mt we confirmed that the level of intact drTRPA1 protein was much reduced in both morph1- and morph3-injected embryos (Fig. 4k).

Small interfering RNAs in mouse

To disrupt *Trpa1* expression in mouse hair cells, we designed two hairpin small interfering (si)RNAs targeting mmTRPA1 (siRNA-6 and siRNA-14; Supplementary Fig. S1a) and placed them under the control of the H1 promoter in the pSUPER vector, which were then inserted into adenoviruses. Although adenoviruses readily infect hair cells, first generation replication-incompetent adenoviruses have sufficient toxicity to damage hair bundles and disrupt transduction²⁷. To avoid such toxicity we developed an adenoviral vector that is multiply deleted of the E1, polymerase and pTP genes²⁸, and that—unlike first generation adenoviruses—does not damage hair-cell transduction even many days after infection^{29,30}.

When mRNA is cleaved by introduction of siRNAs after differentiation, function is not lost until the endogenous protein product has been degraded. We reasoned that we could disrupt TRPA1 function without delay if we infected cells before the TRPA1 channels are normally synthesized. Transduction in hair cells of the mouse utricle normally appears at E17 (ref. 21), so we cultured

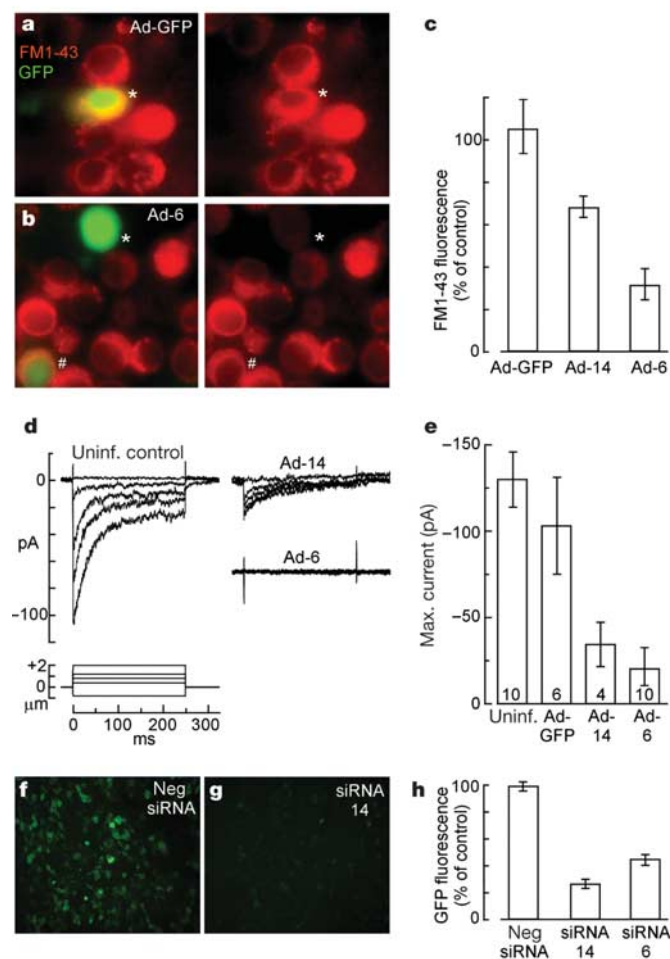


Figure 5 Inhibition of transduction in mouse with adenoviruses encoding siRNAs. **a**, Utricular hair cells labelled with FM1-43 to mark transducing cells; optical section through the cell body. A hair cell (asterisk) infected with Ad-GFP (green) accumulated FM1-43. **b**, Ad-6-infected cells are green; only one (hash) accumulated FM1-43. **c**, Summary of FM1-43 accumulation in infected cells (average intensity per cell) relative to uninjected controls. Data are mean $\pm$ s.e.m. **d**, Typical transduction currents in uninjected cells and cells infected with Ad-14 or Ad-6. **e**, Summary of peak currents. Data are mean $\pm$ s.e.m., N is as indicated. **f**, HEK cells transfected with GFP::mmTRPA1 and a negative control siRNA construct. **g**, HEK cells transfected with GFP::mmTRPA1 and siRNA-14. **h**, Green fluorescence intensity above background, with co-transfection with the control siRNA, siRNA-14, or siRNA-6. Data are mean $\pm$ s.d.; $N = 5$.

utricles from mice at E15, infected them with adenoviruses in culture at E16, and studied the cells at E17–E20.

We first asked whether the siRNAs inhibited FM1-43 accumulation. The adenoviruses also encoded GFP and so infected cells were fluorescent and could be compared to adjacent, uninfected control cells. Cells infected with adenoviruses encoding either siRNA (Ad-6 and Ad-14) displayed diminished FM1-43 fluorescence compared with controls, but cells infected with the same adenovirus encoding GFP alone (Ad-GFP) did not (Fig. 5a–c).

With whole-cell patch clamping, we then recorded the transduction current from infected and control cells. Uninfected cells had transduction currents of 100–150 pA, and cells infected with Ad-GFP had transduction currents not significantly lower (Fig. 5d, e). However, cells infected with adenoviruses encoding siRNAs had currents significantly smaller than either uninfected or Ad-GFP-infected controls ($P < 0.04$; Fig. 5d, e). We did not detect a change in other aspects of the response, for instance in the time course of adaptation or the resting position of the activation curve. Voltage-activated currents in Ad-6- and Ad-14-infected embryonic cells were indistinguishable from those in uninfected controls, suggesting that the siRNAs primarily reduced the number of functional transduction channels. As a further control for toxicity, we cultured utricles from P3 mice infected with Ad-6, and recorded from infected cells 1–2 days after infection. We reasoned that any adenovirus toxicity would be apparent in this time frame, but that the endogenous TRPA1 protein would not be significantly degraded. Infected cells had a mean transduction current equivalent to uninfected controls at that age (165 ± 29 pA, mean $\pm$ s.e.m., $N = 4$), and their current–voltage relations and adaptation were also similar to controls.

Finally, we confirmed that the siRNAs reduce the expression of *Trpa1*. We transfected HEK cells with a plasmid encoding a GFP::mmTRPA1 fusion protein driven by a CMV promoter, and co-transfected these cells with plasmids encoding siRNA-6, siRNA-14, or a negative control siRNA sequence. Green fluorescence, indicating expression of the GFP::mmTRPA1 fusion, was significantly reduced when cells were co-transfected with either siRNA-6 or siRNA-14 compared with the control siRNA. In infected utricles, the low infection rate precluded analysis by immunoblot or RT-PCR; however, we often observed that hair cells infected with Ad-6 had little or no TRPA1 immunoreactivity compared with uninfected neighbours (Supplementary Fig. S2b).

Discussion

A variety of evidence supports the idea that TRPA1 is a component of the mechanosensitive transduction channel of vertebrate hair cells. The level of TRPA1 mRNA increases markedly in the mouse utricle at E17, the same developmental stage at which transduction appears in utricular hair cells. Antibodies to TRPA1 label the stereocilia, kinocilia and the pericuticular zone of hair cells in both bullfrog and mouse. Although antibody label was not always strong in stereocilia of mouse hair cells, it did match immunoreactivity reported for two other components of the transduction apparatus—cadherin 23 and myosin 1c—which are at the tips of stereocilia but also present throughout kinocilia^{23,24}. Moreover, labelling disappeared from the hair bundles within minutes during treatment with La^{3+} or the Ca^{2+} chelator BAPTA, which break tip links and cause similar redistribution of the tip-link protein cadherin 23 (ref. 24).

Additional support comes from inhibiting *Trpa1* expression in zebrafish and mouse. Two different morpholino oligonucleotides directed against zebrafish TRPA1 reduced transduction channel function, as measured both with accumulation of FM1-43 and with microphonic potential responses. Morpholino-injected fish had nearly normal numbers of hair bundles, arguing against an indirect developmental effect. In mouse utricle, two different adenoviruses encoding siRNAs targeting TRPA1 message resulted in decreased

FM1-43 accumulation and decreased transduction currents. These siRNA effects were not attributable to adenovirus toxicity. Finally, the transduction channel of turtle and mouse hair cells is blocked by 5–10 μM of ruthenium red (ref. 31; J.R.H., data not shown), similar to the block of TRPA1 channels expressed in bullfrog oocytes. It seems most likely that TRPA1 is a component of the hair-cell transduction channel itself.

What is the role of the TRPN1 (or NOMPC) channel, which is also necessary for hair-cell transduction in zebrafish¹⁵? Perhaps TRPA1 and TRPN1 form heteromultimeric channels in zebrafish, with both necessary for transduction. Perhaps, instead, individual hair cells use either TRPA1 or TRPN1, but not both. Whatever the case in zebrafish, TRPA1 is the only good candidate for the mechanosensitive transduction channel of mammalian hair cells, as the genomes of mammals lack TRPN1 (data not shown).

TRPA1 is also reportedly activated by cold and by compounds such as horseradish, mustard oil, cinnamon oil and cannabinoids^{16–18}. The variety of agonists suggests a number of receptors coupled to TRPA1 by second messengers; the mechanical activation of the hair-cell transduction channel is, however, far too fast to involve a second messenger³. Many small-diameter neurons in dorsal root ganglia express TRPA1, suggesting a role in chemical nociception. Perhaps TRPA1 in sensory neurons is activated by a variety of painful stimuli, both indirectly through associated receptors and directly by strong mechanical force.

TRPA1 and TRPN1 (NOMPC) are unique among the TRP channel superfamily in possessing a large number of ankyrin domains in their amino termini (17 and 29, respectively). Moreover, the TRPA and TRPN branches of the family are not closely related phylogenetically¹³, and the ankyrin domains of each are no more similar to each other than they are to the multiple ankyrin domains of the three ankyrin genes. This argues for elaboration of the ankyrin domains in these two TRPs as a case of convergent evolution, and begs the question of what special function they might serve.

The ankyrin repeats might bind to motor proteins that maintain tension on the channel³². If so, we might expect the loop regions of each repeat to be very similar if they bind to many copies of the same motor; instead, the loop sequences are the least conserved part of the ankyrin repeats within TRPA1. Alternatively, the ankyrin repeats might bind other signalling or modulatory proteins. If TRPA1 is most generally a polymodal nociceptor, different receptor proteins could dock at the extended N terminus in different cellular contexts.

Most intriguing, however, is the possibility that tandem ankyrin repeats can form a spring (V. Bennett, personal communication; see also refs 33, 34). Hair-cell transduction channels are in series with a biophysically defined “gating spring” of $\sim 1 \text{ mN m}^{-1}$ stiffness³⁵. The tip link has been considered to be the gating spring; however, the recent discovery^{24,36} that the tip link is composed in large part by cadherin 23, which is probably not very elastic, shifts attention elsewhere³³. The crystal structure of ankyrin repeats suggests another possibility: the two helices of each repeat pack pairwise to form an extended but curved structure³⁷. Molecular dynamics simulations (M. Sotomayor, K. Schulten and D.P.C., unpublished results) suggest that its curvature creates an effective stiffness of a few mN m^{-1} , about the same as that needed for the gating spring. Moreover, they suggest that larger forces can pull apart the packed helices, perhaps serving as a safety release to prevent damage by excessive stimuli. If the ankyrin repeats of TRPA1 form a spring, the tip link could be a relatively stiff linker while the elastic gating spring is intracellular and part of the channel itself³⁸.

TRPA1, if it is the hair-cell transduction channel, may serve another role in hearing: after channel opening, Ca^{2+} enters and binds to the channel or a nearby site to promote channel closure, a process termed fast adaptation^{35,39}. The rapid conformational change associated with channels closing together tightens tip links and moves the hair bundle by several nanometres. It has been suggested that this mechanism, if timed appropriately to different

stimulus frequencies for each hair cell, could amplify the vibration of the cochlea's basilar membrane (a process termed the 'cochlear amplifier') and so mediate frequency tuning for auditory sensation⁴⁰. If so, the TRPA1 protein could be at once the hair-cell transduction channel, the gating spring and the cochlear amplifier. □

Methods

Full methods are available in Supplementary Information.

In situ probes

Segments of cDNA for each TRP channel were amplified by PCR from mouse libraries and cloned into the pCRII-TOPO vector (Invitrogen). TRP channel cDNA flanked by Sp6 and T7 promoters was amplified and used for *in vitro* transcription with digoxigenin-labelled rUTP. Hybridizations on mouse E17 and P0 frozen sections were visualized with anti-DIG F_{ab} conjugated to alkaline phosphatase.

Real-time RT-PCR

Twenty utricular epithelia were excised from embryonic mice, from E14 to E19. DNase-free RNA was prepared (RNAqueous-4PCR; Ambion) and used to make cDNA with TaqMan reagents (Applied Biosystems). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and an I-Cycler (Bio-Rad). DNase-treated RNA was a negative control and melt curve analysis confirmed that a single product was amplified. Gene expression was normalized to myosin 7a; normalization to S29 gives similar results.

Antibody generation and labelling

Rabbits were immunized with a GST fusion of mouse TRPA1 C terminus. Antibody 0203mt was purified on the mouse C terminus and 0203f1 on a conserved peptide within the zebrafish TRPA1 C terminus. Bullfrog sacculles were dissected and their otolithic membranes removed, then either fixed immediately or incubated for 20 min with 5 mM La³⁺, 5 mM BAPTA, or 0.1 Ca²⁺ before fixation. Cochleae, utricles and semicircular canals from neonatal mice were surgically exposed and fixed *in situ*. Primary antibodies were used at 7–10 µg ml⁻¹.

Zebrafish TRPAs

Fragments of two zebrafish homologues of mouse *Trpa1* were identified in the Ensembl database. RNA was collected from 4–5 d.p.f. zebrafish and cDNA made using SMART RACE cDNA amplification (BD Biosciences). Primers for full-length zebrafish *trpa* genes were based on RACE data and on Ensembl sequence. Exon structures were determined by comparison to genomic sequence in the Ensembl database. The two zebrafish homologues of mouse *Trpa1* are deposited in GenBank under accession numbers AY677196 and AY677197.

Morpholino injections and RT-PCR

Morpholinos targeting drTRPA1, drTRPA2 or a standard control (Gene Tools) were injected into one-cell-stage zebrafish embryos (~2.5 nl; 0.5 mM stock). Embryos were studied at 58–70 h.p.f. Total RNA from 60–h.p.f. embryos was extracted by Trizol, genomic DNA removed by DNase I, and cDNA synthesized by Superscript II (Invitrogen). Nested PCR amplified a drTRPA1 fragment from exon 18 to exon 22, which was sequenced.

Immunoblots

Proteins from morphant embryos (30–40 animals; 58–60 h.p.f.) or four bullfrog eyes were run on a 10–20% gradient SDS gel. DrTRPA1 was detected with antibody 0203mt (1:750) and HRP-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) and visualized with enhanced chemiluminescence (Amersham).

Microphonic recording

Larval zebrafish (48–72 h.p.f.) were anaesthetized and mounted dorsal side up. Bath saline contained tetrodotoxin to reduce twitching. Recording pipettes (2–6 MΩ) were advanced through the swelling near the lateral semicircular canal and placed near the otolith of the posterior macula. Microphonic potentials were elicited with a probe placed just behind the otocysts and vibrated at 5–100 Hz along the rostral–caudal axis. A similar method was recently described⁴¹.

FM1-43 and actin labelling

To label lateral line hair cells, embryos (58–60 h.p.f.) were incubated in AM1-43 (5 µM; 5 min), washed, fixed and photographed with a fluorescence microscope. For quantification, pixel intensity was integrated over each neuromast.

To label inner ear hair cells after microphonic recording, recording pipette solution (containing 40 µM FM1-43; Molecular Probes) was pressure-injected into the otocyst. Dye entered hair cells and extracellular dye diffused from the otocyst for 5–10 min before confocal imaging. Embryos were fixed and permeabilized with acetone to extract residual FM1-43 before labelling with fluorescent phalloidin for additional confocal imaging.

siRNAs

siRNA oligonucleotides, designed for RNA hairpin formation, were cloned into the pSUPER vector under an H1 RNA promoter (OligoEngine). A sub-fragment of the vector was subcloned into the pAdTrack shuttle vector⁴²—for H1-driven siRNA expression and CMV-driven GFP expression—which was recombined with the pAdEΔpol, ΔpTP plasmid

(derived from pAdEasy⁴², but lacking parts of the polymerase gene and the pTP gene) to generate pAdEΔpol, ΔpTP/siRNA + GFP, which was grown in *trans*-complementing C-7 cells²⁸. Utricles dissected at E15 (ref. 21) were incubated at 37 °C and exposed to adenoviral vectors for 4–24 h, then washed and maintained at 37 °C for 2–5 days.

Recording and fluorescence imaging in mouse utricle

Utricular hair-cell stimulation and recording were previously described²¹. Infected cells were identified by GFP fluorescence and photographed, FM1-43 was applied (5 µM, 10 s) and cells were photographed using a TRITC filter set.

GFP::TRPA1 expression construct and siRNA testing

Full-length mmTRPA1 cDNA was cloned into the N-terminal GFP TOPO vector (Invitrogen) to generate the plasmid pcDNA3.1GFP::TRPA1. HEK293 cells were co-transfected with this plasmid and with siRNA constructs (Ambion Silencer Express) containing a mouse U6 promoter directing transcription of either a negative control sequence or hairpin sequences corresponding to siRNA-6 and siRNA-14. GFP::TRPA1 expression in transfected cells was assessed 24–48 h after transfection. Total fluorescence in a field was calculated from average pixel intensities.

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Crystalline water ice on the Kuiper belt object (50000) Quaoar

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The Kuiper belt is a disk-like structure consisting of solid bodies orbiting the Sun beyond Neptune¹. It is the source of the short-period comets and the likely repository of the Solar System's most primitive materials². Surface temperatures in the belt are low (~ 50 K), suggesting that ices trapped at formation should have been preserved over the age of the Solar System. Unfortunately, most Kuiper belt objects are too faint for meaningful compositional study, even with the largest available telescopes. Water ice has been reported in a handful of objects^{3–5}, but most appear spectrally featureless^{5,6}. Here we report near-infrared observations of the large Kuiper belt object (50000) Quaoar, which reveal the presence of crystalline water ice and ammonia hydrate. Crystallinity indicates that the ice has been heated to at least

110 K. Both ammonia hydrate and crystalline water ice should be destroyed by energetic particle irradiation on a timescale of about 10^7 yr. We conclude that Quaoar has been recently resurfaced, either by impact exposure of previously buried (shielded) ices or by cryovolcanic outgassing, or by a combination of these processes.

Object (50000) Quaoar (formerly 2002 LM60) is, after Pluto, the largest and brightest known Kuiper belt object. Its diameter and red geometric albedo are $1,260 \pm 190$ km and $0.09^{+0.04}_{-0.02}$, respectively⁷. Quaoar therefore constitutes a particularly attractive target for spectroscopy in search of exposed volatiles. We observed Quaoar using the CISCO spectrometer⁸ at the Subaru 8-m telescope, on UT 2004 May 8 and 9. Quaoar had heliocentric distance 43.37 AU, geocentric distance 42.46 AU and phase angle 0.58° . The apparent visual magnitude was approximately $V = 18.5$.

The resulting albedo spectrum is plotted in Fig. 1, including data from three infrared grating settings as well as an optical spectrum⁹, all normalized to a red geometric albedo $p_R = 0.09$ (ref. 7). Inclusion of the optical spectrum gives continuous wavelength coverage from 0.4 to $2.5 \mu\text{m}$. The unsmoothed spectrum is shown in Fig. 1 as a black line. Superimposed as a red line is a lightly smoothed spectrum to guide the eye. Regions of high noise are seen near 1.4, 1.9 and longward of $2.4 \mu\text{m}$, corresponding to telluric absorption by atmospheric water vapour.

The most important spectral features in Quaoar include a steep, positively sloped continuum from 0.4 to $\sim 1.3 \mu\text{m}$ wavelength (Fig. 1). This is characteristic of a strong ultraviolet absorber, probably associated with an organic surface composition¹⁰. Additional absorption bands at 1.5 and $2.0 \mu\text{m}$ (Fig. 2) indicate water ice, while a band at $1.65 \mu\text{m}$ is characteristic of crystalline (as opposed to amorphous) structure in the ice¹¹. Spectra of other Kuiper belt objects in which water is detected have lacked the quality needed to show the $1.65\text{-}\mu\text{m}$ band^{3,4,5}. Comparison of the pure-

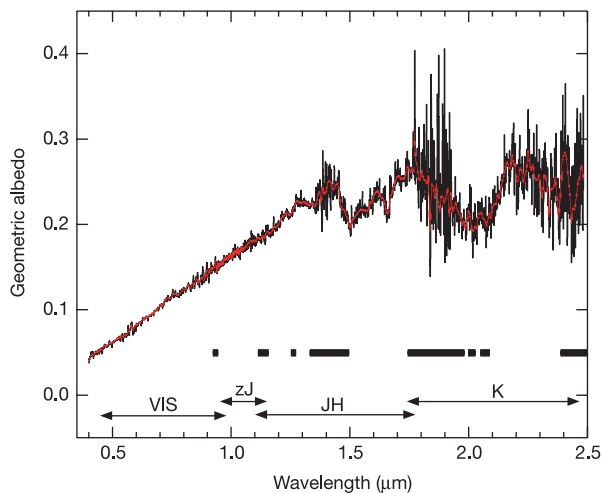


Figure 1 Albedo spectrum of Quaoar showing red optical continuum and distinct absorption bands at 1.5, 1.65 and $2.0 \mu\text{m}$. Spectra from four gratings (VIS is from ref. 9, zJ, JH and K are different CISCO grating settings) were spliced together using regions of spectral overlap (see Table 1) and normalized to the R -band geometric albedo of 0.09 at $0.65\text{-}\mu\text{m}$ wavelength⁷. Uncertainty in the levels of the different segments of the spectra is $\sim 5\%$ or less. The black line shows the unsmoothed data. The red line shows a running box-average with a smoothing width of $100 \text{ \AA}$ in VIS, $40 \text{ \AA}$ in zJ, $140 \text{ \AA}$ in JH and $140 \text{ \AA}$ in K. Wavelengths at which the atmospheric transmission is $\leq 80\%$ are marked with horizontal bars. CISCO uses a $1,024 \times 1,024$ pixel HgCdTe 'HAWAII' infrared array as detector with image scale $0.105 \text{ arcsec pixel}^{-1}$, and field width 108 arcsec . Quaoar was acquired by imaging with the grating removed from the spectrograph and confirmed from its proper motion relative to background objects. The spectrograph slit was aligned parallel to the proper motion and the telescope guided at sidereal rates. Seeing on both nights was near 0.4 arcsec at J and $0.3\text{--}0.4 \text{ arcsec}$ at K, allowing us to use a 0.6-arcsec -wide slit. Individual integration times were 300 s for the zJ and JH spectra, and 250 s for the K spectra: image trail during the integrations was less than 0.2 arcsec . For background subtraction, the spectra were taken in pairs while dithering the image along the slit by 10 arcsec . Approximately every 1.5 h we observed A-type and G-type calibration stars within 0.05 airmasses of Quaoar and, following visits to the stars, we re-centred Quaoar in the slit. Spectra of A-type stars were used to cancel telluric features. Spectra of G-type stars were used to remove the colour of the Sun. The atmosphere was stable with a precipitable water column of $\sim 2 \text{ mm}$. Spectra were flattened, sky-subtracted, traced and extracted using a 1.05-arcsec -wide aperture.

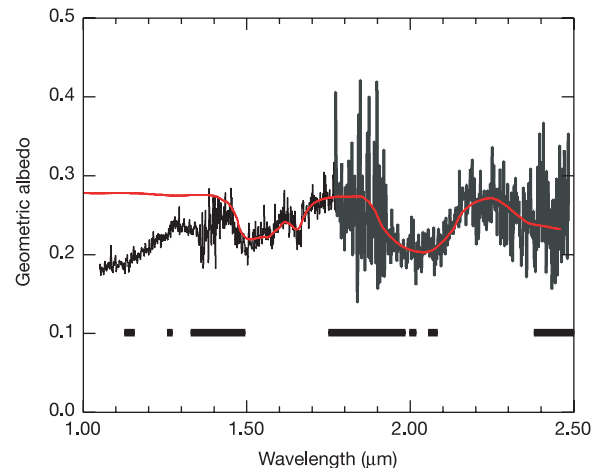


Figure 2 Near-infrared reflection spectrum of Quaoar (black) compared with a water-ice reflection spectrum¹⁰ (red). The water spectrum has not been fitted to the data except for vertical scaling to match the depth of the $2\text{-}\mu\text{m}$ band. Wavelengths at which the atmospheric transmission is $\leq 80\%$ are marked with horizontal bars. In the 0.4 to $0.95\text{-}\mu\text{m}$ region, the slope normalized to $0.65 \mu\text{m}$ is $S'_{0.65} = 28 \pm 2\%/1,000 \text{ \AA}$ (ref. 9). In our zJ spectrum (0.89 to $1.10 \mu\text{m}$) the slope normalized in the same way is $S'_{0.65} = (27 \pm 1\%/1,000 \text{ \AA})$, whereas at longer wavelengths the reddened continuum becomes neutral. Broad absorption bands centred near 1.5 and $2.0 \mu\text{m}$ unambiguously reveal the presence of water ice on Quaoar. A third, weak water-ice band near $1.3 \mu\text{m}$ (ref. 11) may be evident in the spectrum but is partially overlapped by a telluric feature. The band depths (22% for the $2.0\text{-}\mu\text{m}$ band) and the geometric albedo outside the bands both suggest that the ice is impure, or that ice occupies only a small part of the total surface of (50000) Quaoar. A relatively narrow ($0.015 \mu\text{m}$) absorption near $1.65 \mu\text{m}$ is characteristic of cold, crystalline water ice and is specifically not present in pure, amorphous ice^{11,12}.

water-ice spectrum with the data reveals a more subtle feature near $2.2\text{ }\mu\text{m}$ (Fig. 3) which coincides with a band due to ammonia hydrate ($\text{NH}_3\cdot\text{H}_2\text{O}$)^{12,13}. At 40 K, the 1.5 and $2.0\text{-}\mu\text{m}$ bands have peak absorption coefficients of ~ 50 and $\sim 100\text{ cm}^{-1}$, respectively¹², corresponding to photon penetration depths in ice of about 0.2 and 0.1 mm. The spectrum therefore samples ice in the very uppermost layers on Quaoar.

A search for other plausible ices was unsuccessful. The spectrum is unlike that of Pluto (compare with Fig. 4), which is dominated by CH_4 and which also shows solid N_2 and CO (ref. 14). Our ability to detect N_2 and CO is questionable, given the spectral resolution of our data, but the eight strong bands of CH_4 in the 1.0 to $2.4\text{ }\mu\text{m}$ spectral region are conspicuously absent in Quaoar, eliminating any possibility that the $1.65\text{-}\mu\text{m}$ feature is methane-contaminated. CO_2 is not present either, down to the level of the noise in the spectrum. Quaoar is spectrally much more similar to Charon, in showing strong water-ice bands and a weak absorption due to ammonia hydrate^{15,16}. The absence of other molecules is unsurprising given the small size and low ($\sim 500\text{ m s}^{-1}$) escape velocity of Quaoar.

The detection of crystalline ice on Quaoar shows that the temperature has exceeded the critical $T_c \approx 105\text{--}125\text{ K}$ range for crystallization (see Fig. 1 of ref. 17). The radiation equilibrium temperature (for albedo ~ 0.1 and distance $\sim 43\text{ AU}$) is only $\sim 50\text{ K}$. Temperatures higher than T_c could be generated through radioactive decay heating in the interior of Quaoar¹⁸ but not on the surface, where the temperature is limited by radiation to space. Impact heating by micrometeorites could be responsible for heating surface ice above the crystallization temperature. However, the vapour pressures of ammonia and water are very different, and

impact heating is expected to lead to the progressive loss of ammonia from the surface¹⁹. Impact heating thus appears at odds with the presence of ammonia hydrate unless this can be resupplied by another process.

A further complication arises from the instability of crystalline ice and ammonia hydrate to energetic particle bombardment. At low temperatures, crystalline ice is converted to the amorphous phase by the bond-breaking effects of cosmic rays and solar wind particles^{20,21}. Ammonia hydrate is also unstable to energetic particle bombardment, with a survival time of $<10^6\text{ yr}$ at Saturn¹⁹. The timescale for complete processing of the optically active top ($0.1\text{-}\mu\text{m}$ thick) layer at 40 AU is $\sim 10^7\text{ yr}$ (ref. 22), much less than the age of the Solar System. Persistence of crystalline ice and ammonia hydrate at the surface therefore implies either that the ice is protected (for example, by a magnetosphere or by an atmosphere, neither of which seems likely at Quaoar) or that the observed ice has been replenished within the past 10^7 yr . On Charon, ammonia hydrate could be chemically produced from a constant supply of nitrogen fed to the surface by the escaping N_2 atmosphere

Table 1 Parameters of the spectra

Date	Grating	Wavelength*	Spectral dispersion ($\text{\AA pixel}^{-1}$)	Integration time (s)
08 May	zJ	0.89–1.30	5.83	1,800
09 May	zJ	0.89–1.30	5.83	1,800
08 May	JH	1.05–1.90	8.23	2,400
09 May	JH	1.05–1.90	8.23	2,400
08 May	HK	1.77–2.49	8.63	6,000
09 May	HK	1.77–2.49	8.63	1,500

*Approximate minimum and maximum wavelengths (in μm) recorded in each spectrum.

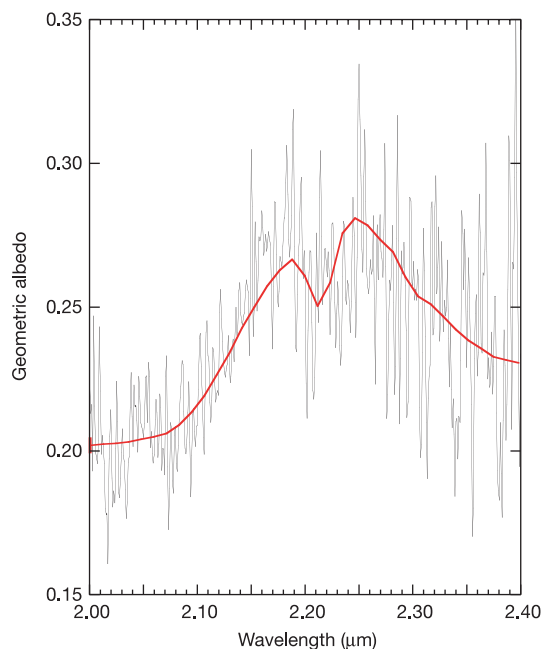


Figure 3 Close-up of the reflection spectrum of Quaoar centred at $2.2\text{ }\mu\text{m}$. Unsmoothed data are shown by a black line. A model of ammonia hydrate $\text{NH}_3\cdot\text{H}_2\text{O}$ (3% ammonia)¹⁶ is overplotted in red. Unlike pure water ice, which has a reflection maximum at $2.2\text{ }\mu\text{m}$ (compare with Fig. 2), both Quaoar and ammonia hydrate show a local minimum at this wavelength. The Quaoar band is centred at $2.220 \pm 0.005\text{ }\mu\text{m}$ and has a depth of about 10% relative to the local continuum. For comparison, the wavelength of the vibrational overtone of N-H in pure ammonia ice is $2.24\text{ }\mu\text{m}$, significantly longer than observed, but in ammonia hydrate ($\text{NH}_3\cdot\text{H}_2\text{O}$) at 65 K the band wavelength is $2.21\text{ }\mu\text{m}$ (refs 12, 13), which overlaps the measured value. The absence of other ammonia hydrate bands (at 1.04, 2.0 and $2.3\text{ }\mu\text{m}$) in our data is consistent with model spectra provided the fractional amount of NH_3 is $\leq 10\%$ (ref. 16). A similar band has been reported at $2.20\text{--}2.22\text{ }\mu\text{m}$ in Pluto's satellite Charon^{15,16} and at $2.21\text{ }\mu\text{m}$ on Uranus' satellite Miranda²³.

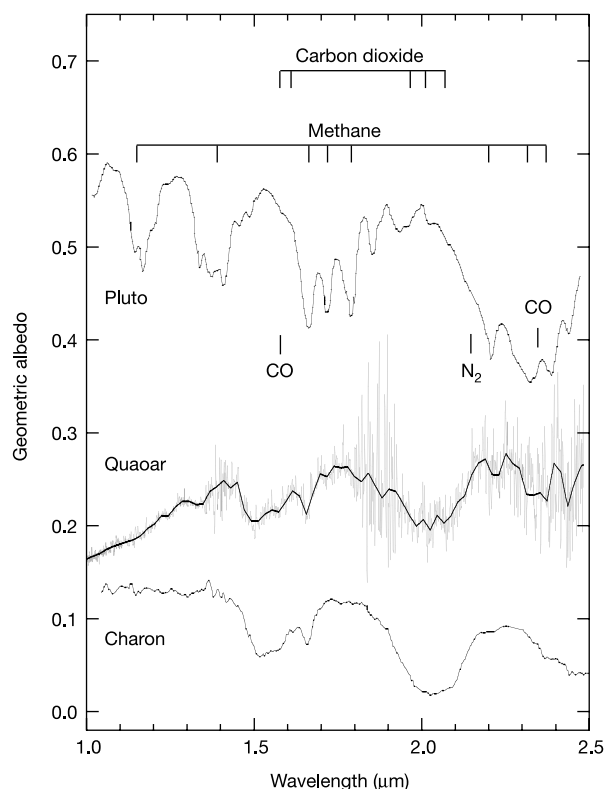


Figure 4 Near-infrared spectra of Pluto, Quaoar and Charon compared. The Pluto and Charon spectra¹⁵ have been displaced vertically and scaled to facilitate comparison with the spectrum of Quaoar. Wavelengths of absorption bands of solid CO , CO_2 , N_2 and CH_4 ice are indicated.

of nearby Pluto¹⁶. There is no corresponding external source of nitrogen at Quaoar (or Miranda²³), and an internal source must be presumed.

Impacts into the surface could mix unirradiated (ammonia-rich, crystalline) subsurface material with the irradiated surface ice. Alternatively, or perhaps in combination with impact 'gardening', subsurface material could be exposed by continued outgassing onto the surface as a result of cryovolcanism. Complex geology on small icy satellites, including several smaller than Quaoar, provides pervasive evidence for solid-state convection that could bring warm, ammoniated ice towards the surface²⁴. The energy sources driving this activity are not fully understood, but are likely to include radioactivity and tidal heating (in planet-satellite systems). Ammonia hydrate is an expected product of condensation in the high-density protoplanetary subnebulae, and evidence from the comets, where the ammonia abundance is ~1%, shows that this molecule is also trapped from the solar nebula²⁵. It may play a central role in satellite thermal evolution and geology by decreasing the viscosity and enabling the flow of ice²⁶. Quaoar is certainly large enough to be cryovolcanically active and the crystalline ice and ammonia hydrate are consistent with the products of such activity. □

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Routing of anisotropic spatial solitons and modulational instability in liquid crystals

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In certain materials, the spontaneous spreading of a laser beam (owing to diffraction) can be compensated for by the interplay of optical intensity and material nonlinearity. The resulting non-diffracting beams are called 'spatial solitons' (refs 1–3), and they have been observed in various bulk media^{4–6}. In nematic liquid crystals^{7–9}, solitons can be produced at milliwatt power levels^{10–12} and have been investigated for both practical applications¹³ and as a means of exploring fundamental aspects of light interactions with soft matter^{14,15}. Spatial solitons effectively operate as waveguides, and so can be considered as a means of channelling optical information along the self-sustaining filament. But actual steering of these solitons within the medium has proved more problematic, being limited to tilts of just a fraction of a degree^{16–20}. Here we report the results of an experimental and theoretical investigation of voltage-controlled 'walk-off' and steering of self-localized light in nematic liquid crystals. We find not only that the propagation direction of individual spatial solitons can be tuned by several degrees, but also that an array of direction-tunable solitons can be generated by modulation instability^{21–25}. Such control capabilities might find application in reconfigurable optical interconnects, optical tweezers and optical surgical techniques.

Previously investigated angular steering of spatial solitons (SSs) has been limited to very small angles, typically fractions of a degree^{16–20}. There are two reasons for this, one applying to theoretical models, and the other to material systems. First, there is an intrinsic limitation associated with the frequently invoked paraxial approximation. The latter allows the understanding of most SS physics, but its validity is limited to wavepackets localized on-axis with respect to the source light beam. Large-angle steering is ruled out by such treatment, if used in a consistent way. Second, the most studied SSs in bulk matter (such as 2D + 1 parametric and photorefractive solitons^{26,27}) require propagation in anisotropic crystals; in actual arrangements, such material complexity places severe constraints (for example, phase-matching in parametric SSs and anisotropic charge drift/diffusion in photorefractive SSs) on the experimental observation of SSs with adjustable directions of propagation. Hence, a number of simplifying assumptions have

been adopted to describe light propagation at limited (typically fractions of a degree) angles with respect to crystallographic axes^{1–3}. Current models for SSs assume rather small, or even vanishing, ‘walk-off’ angles (that is, angles between the ordinary (‘o’) and extraordinary (‘e’) Poynting vectors excited in a birefringent (in the simplest case, uniaxial) crystal). With reference to Poynting vector(s), although the ordinary wave propagates parallel to the (wavevector of the) entering beam, the extraordinary one is tilted by an amount depending on crystal orientation and degree of anisotropy (see below). The two beams are associated with distinct wavenumbers and refractive indices, their difference being the so-called birefringence.

Another fundamental process in which walk-off has been neglected is spatial modulational instability^{21–25}. Such instability is often associated with the observation of SSs, the latter being nonlinear attractors in the system dynamics^{1–3}. In fact, modulational instability is at the origin of the breakup of a wide beam into filaments, as observed in a self-focusing optical medium (see below) when excited by a plane-wave-like excitation. Eventually, modulational instability can produce a set of uniformly spaced spatial solitary waves. For similar reasons as those discussed above for SSs, the effects of large birefringence on modulational instability have not been investigated. We show below that, when dealing with the existence of an extraordinary beam (considering a paraxial beam in

a rotated frame with respect to the input direction), the above difficulties can be superseded, allowing complete understanding and the demonstration of large-angle voltage-controlled dragging of both individual SSs and modulational instability patterns.

The sample we used is the nematic liquid crystal (NLC) E7, confined by capillarity in a planar cell. Two treated glass slides are spaced by 75 μm and held parallel to each other with the aid of two thin Kapton strips. A third slide seals the input air/NLC interface, avoiding lensing and depolarization due to the meniscus otherwise formed at the entrance (Fig. 1). Conductive indium-tin-oxide films on top and bottom slides enable the application of a low-frequency bias (1 kHz), while rubbed polymer coatings induce planar alignment of the NLC molecular director (unit vector $\hat{n}$) at $\xi = 45^\circ$ with respect to the light-beam ‘external’ direction of propagation (that is, input wavevector). Using a 20 $\times$ microscope objective, light from a Nd:YAG laser ($\lambda = 1,064 \text{ nm}$) is coupled into the cell with wavevector $\mathbf{k}$ parallel to the z -axis (the corresponding unit vectors are denoted $\hat{k}$ and $\hat{z}$, respectively). A linear polarizer and a half-wave plate control the input power and polarization, while the beam evolution is monitored by collecting the light scattered above the cell (from the plane y – z) with an optical microscope and a high-resolution CCD camera.

Figure 2 shows a typical retrieved intensity distribution in the plane y – z for a beam excitation with no specific polarization (that is containing both ‘e’ and ‘o’ eigenwaves). At low power ($< 100 \mu\text{W}$), the propagation of ordinary (o-) and extraordinary (e-) components in the beam is apparent (Fig. 2a). The former is parallel to the input $\mathbf{k}$ -vector; the latter appears to travel at angle α , in general differing from the walk-off δ owing to the 3D projection of the

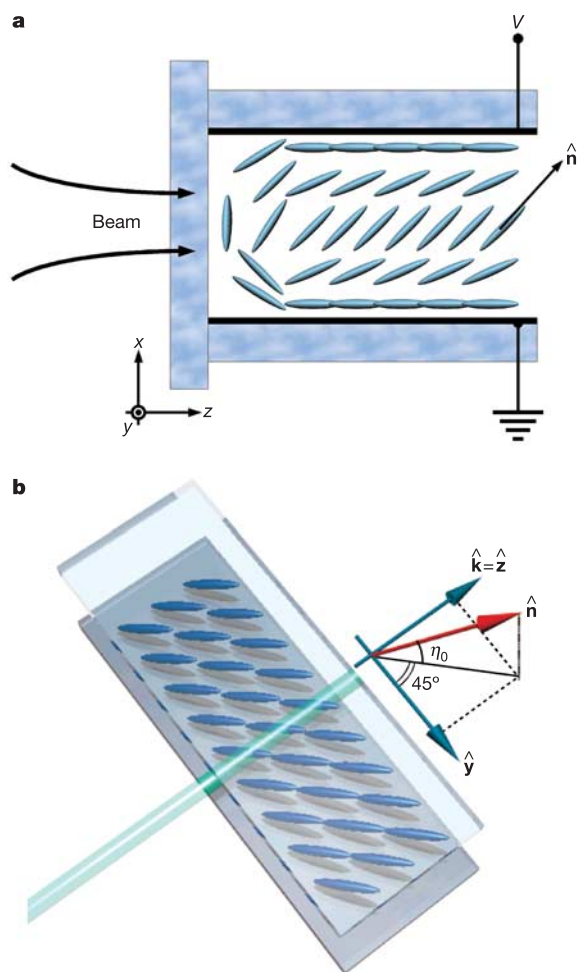


Figure 1 Sketch of the sample. **a**, Side view, **b**, 3D view, with an indication of the input beam. NLC molecules, with large-scale order given by the director $\hat{n}$, are planarly anchored in the y – z plane and aligned at 45° with respect to the input interface. The elevation angle is $\eta_0 \approx 0$ in the absence of bias ($V = 0 \text{ V}$) and approaches 90° for $V \approx 3 \text{ V}$.

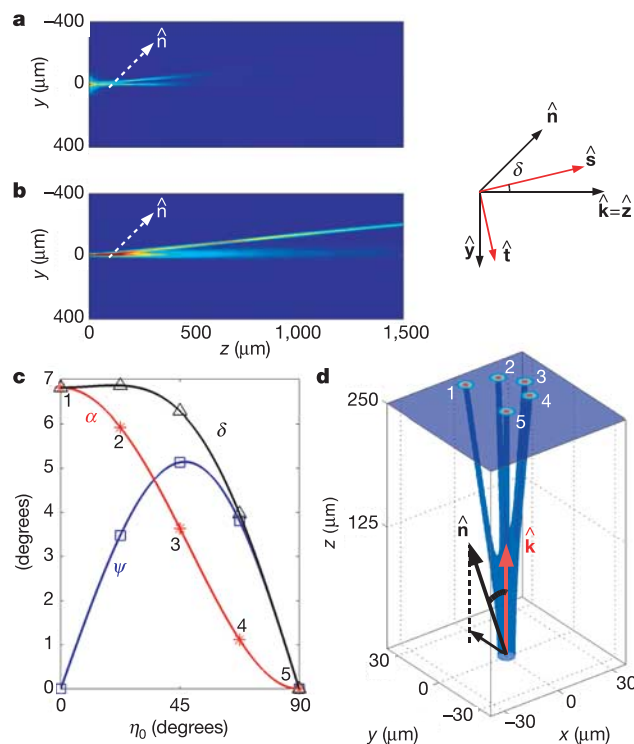


Figure 2 Spatial soliton voltage routing. **a**, **b**, Ordinary and extraordinary wave at zero bias ($\alpha = \delta$). **a**, At low power both components diffract: the e-beam propagates along $\hat{s}$ (see sketch on right); the o-wave along $\hat{z} = \hat{k}$. **b**, At high power, the e-wave generates a SS. **c**, Calculated walk-off δ and its projected values α and ψ . **d**, Sketch of the solitary beam inside the cell. $\hat{n}$ is the NLC director in the un-biased cell. The SS spot, at $z = 250 \mu\text{m}$, spans a semicircular path (from points 1 to 5, see markers in **c**) as the bias increases.

e-wave on the y - z plane. In our geometry, the walk-off plane, defined by $\hat{z}$ and the molecular director $\hat{n}$, is not parallel to the y - z plane if $V \neq 0$ V. In the following, we denote α the ‘apparent’ walk-off. Both α and δ are linked to the applied voltage, which determines the orientation angle η_0 of the NLC director in the middle of the cell. Note that, as the Rayleigh range is of the order of $30\ \mu\text{m}$ (nearly two orders of magnitude shorter than the length over which SSs remain self-confined), the two components rapidly diffract. For a high power of 5 mW in each wave (Fig. 2b), the molecules tend to align to the input beam polarization, thus increasing the (e-) refractive index and yielding self-focusing. The o-wave is polarized orthogonally to $\hat{n}$, hence re-orientation is inhibited below the so-called Freedericks threshold, nearly an order of magnitude higher than the power levels employed in this work^{7–9}. Therefore, only the e-wave undergoes self-confinement with no diffraction, forming a guiding filament or spatial soliton.

We repeated the experiments for various voltages and observed the effect to be rather robust (see Fig. 3 below), the main feature being the change in direction of the propagating SS: the apparent walk-off α decreases with bias, from 7° to 0° . A movie clip shows such soliton steering versus bias (Supplementary Video 1). As only the e-wave contributes to the soliton, we rotate the input polarization as the bias increases in order to maximize the portion of excitation trapped in the SS. The applied voltage adjusts the input polarization component sustaining the soliton. In other words, the local principal axes rotate with V relative to the laboratory frame of reference: this is a remarkable difference when compared to previously investigated SSs in anisotropic media (such as parametric and photorefractive SSs), where self-confinement is attained for a critical polarization determined by crystallographic orientation.

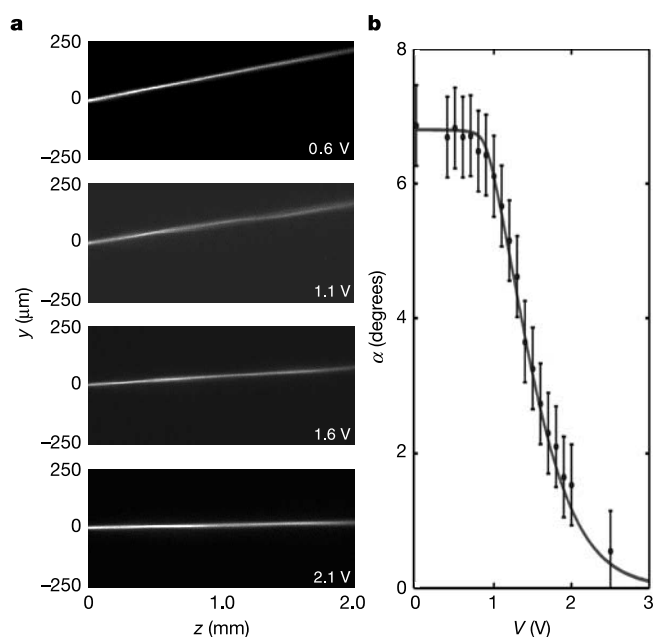


Figure 3 Spatial soliton trajectories and walk-off. **a**, Acquired images of 5 mW solitons propagating at different biases. **b**, Apparent SS walk-off versus bias: data (dots with error bars) and theoretical prediction (solid line). α was evaluated at early stages of propagation, so that beam interactions with the cell boundaries could be ignored. Error bars relate to the pixel size of the CCD camera. The parameters adopted are those for E7 at $\lambda = 1,064\ \text{nm}$ (ref. 30). (The elastic constants for splay and twist, $K_1 = 12\ \text{N}$ and $K_3 = 19.5\ \text{N}$, respectively, were individually accounted for in the low-frequency model, see equations in Supplementary Material, with $n_o = 1.50$, $n_e(\theta = 90^\circ) = 1.70$, $\Delta\epsilon_{\text{LF}} = 14.5$ and $\epsilon_{\text{LF}}^{\perp} = 5.1$.)

The actual walk-off angle δ can be unambiguously related to α by simple geometric considerations. Using well known expressions for the dielectric tensor in NLCs, δ can be linked to η_0 , this being determined by the voltage V applied across $\hat{x}$ (see also the equations in Supplementary Material). η_0 goes from 0° (when $V = 0$ V) to 90° (when V reaches a saturation value close to 3 V). Figure 2c shows the resulting trend in terms of the relevant angles. Owing to the rotation of the walk-off plane, the projection angle ψ in the x - z plane increases, reaching a maximum for $\eta_0 \approx 45^\circ$ and vanishing for large biases $V \approx 3$ V. As visible in Fig. 2d, following the e-wave the self-trapped beam describes a semi-circular trajectory inside the cell, propagating at angle δ with respect to $\hat{k}$. With our imaging system, we acquired the projection of such trajectory on the y - z plane, corresponding to the apparent walk-off α .

Figure 3 shows experimental results for various biases, demonstrating the voltage-controlled soliton steering over a large angular interval. The excitation is 5 mW, and the input waist about $5\ \mu\text{m}$. As expected, the walk-off decreases as the bias increases until, for high biases $V \approx 2.7$ V, reorientation begins to saturate, the nonlinearity becomes less effective and light propagates in a quasi-linear fashion with no walk-off (see Supplementary Figs 1 and 2 in Supplementary Material for additional experimental results).

In order to provide a clear assessment of the phenomenon, it is necessary to develop a theory supporting the existence of stable solitons propagating in the walk-off direction, without limits to the degree of anisotropy. It is also necessary to provide a quantitative model to describe the dependence of (measured) α on V , and compare it with experimental data.

Once the direction of propagation of the input laser beam $\hat{k} = \hat{z}$ is fixed, the theory can be developed using a perturbative expansion of Maxwell's equations. The paraxial approximation along z must be avoided, and the solution can be taken as a superposition of two wavepackets around e- and o-rays (details in equations in Supplementary Material), associated with refractive indices $n_e(\eta_0)$ and n_o (the ordinary index does not depend on molecular orientation), respectively. The evolution equation for the e-wave E , after a ‘multi-scale expansion’ of Maxwell's equations²⁸, turns out to be:

$$2ik_0n_e(\eta_0)\cos[\delta(\eta_0)]\frac{\partial E}{\partial s} + D_t(\eta_0)\frac{\partial^2 E}{\partial t^2} + D_r(\eta_0)\frac{\partial^2 E}{\partial r^2} + k_0^2\Delta\epsilon T(\eta_0)\Phi E = 0 \quad (1)$$

where r is the coordinate orthogonal to the plane $(\hat{t}, \hat{s})$ which rotates with η_0 , $k_0 = 2\pi/\lambda$, $\Delta\epsilon = n_e^2(\theta_0 = 90^\circ) - n_o^2$ measures the dielectric anisotropy, $D_{r,t}(\eta_0)$ are the coefficients of anisotropic diffraction and are given in terms of η_0 and material parameters (see equations and Supplementary Fig. 4 in Supplementary Material). Equation (1) is paraxial along the walk-off direction $\hat{s}$ of the Poynting vector, but not along $\hat{k} = \hat{z}$; therefore, it holds for an arbitrary degree of anisotropy. The nonlinear self-action is linked to the optically induced perturbation Φ of director orientation by $T(\eta_0)$ (in what follows, we take the lowest order in Φ). Equation (2) completes the model of equation (1) by linking Φ to the light intensity, proportional to $|E|^2$ (see equations in Supplementary Material):

$$K\nabla^2\Phi - A(\eta_0)\Phi + \frac{\epsilon_0\Delta\epsilon}{4}\sin[2\theta_0(\eta_0) - 2\delta(\eta_0)]|E|^2 = 0 \quad (2)$$

Here K is the elastic constant of the medium (taken equal for splay, bend and twist), ϵ_0 the vacuum permittivity, and $\theta_0(\eta_0)$ the angle between director $\hat{n}$ and $\hat{z}$. $A(\eta_0)$ is determined by the boundary conditions on the director, hence by the cell geometry; it relates to the decay length R of the optical perturbation far from the beam axis. The ratio between R and the soliton waist quantifies the degree of non-locality^{15,25}. An explicit expression for the case under consideration is given in Supplementary Material.

The system of equation (1) with equation (2) provides the simplest theoretical frame for our observations, and qualifies the observed filaments as non-local anisotropic spatial solitons. SS theory^{1,2,15} permits us to conclude that stable self-trapped beams can travel along the walk-off direction, irrespective of the degree of anisotropy. The model also accounts for a vanishing nonlinearity when—at high voltages—the molecular director $\hat{n}$ is parallel to the polarization $\hat{t}$ of the extraordinary wave ($\eta_0 \approx 90^\circ$), and no further reorientation is possible. Supplementary Fig. 1 in Supplementary Material shows the propagation of an intense beam at high bias, when diffraction takes place because of the substantially reduced nonlinear response.

The quantitative assessment is completed by linking the applied voltage to the director orientation in the middle of the cell (details in the equations in Supplementary Material), and comparing the predicted soliton trajectory with experimental data (see Fig. 3, and Supplementary Fig. 3 in Supplementary Material). The results confirm that the medium supports strongly anisotropic spatial solitons propagating away from the crystallographic axes, at directions controlled by the applied voltage.

An important corollary of the model briefly outlined above concerns transverse modulational instability. When a beam that is wide in the y direction impinges on the NLC cell, its breakup and the formation of multiple filaments can be observed²⁵. In all of the previously reported experimental investigations, including but not limited to NLCs, the role of walk-off was always neglected^{21–25}. Our experimental arrangement allows us to go beyond the latter limit, while our model (equations (1) and (2)) predicts modulational instability at arbitrary walk-off angles with filaments propagating along $\hat{s}$. We verified this experimentally: Fig. 4 shows photographs taken for an input power of 200 mW and a quasi-plane-wave beam 400 μm wide along y . The photographs illustrate the initial stages of filament generation. By varying the applied voltage, angular dragging of the whole pattern is clearly visible. Note that, at low voltages, self-focusing in the beam centre and a pronounced mutual attraction between filaments take place owing to a significant degree of non-locality for small η_0 (corresponding to a reduced $A(\eta_0)$, see

Supplementary Fig. 4 in Supplementary Materials). At high voltages, η_0 saturates and a local behaviour is retrieved. We stress that, in the case of multiple filaments, each SS acts as a waveguide for a co-polarized collinear weak signal, and thus the overall pattern realizes a reconfigurable one-dimensional photonic lattice.

We have reported both experimental evidence and quantitative assessment of the propagation of stable SSs at arbitrary walk-off angles in liquid crystals. The additional observation of modulational instability, to our knowledge the first experimental report of this phenomenon in the presence of strong anisotropy, demonstrates that reconfigurable optically induced waveguide arrays can be not only generated²⁹, but also routed. These findings lend themselves to potential applications and to fundamental advances in the interplay between effects such as anisotropy, nonlinearity and non-locality. $\square$

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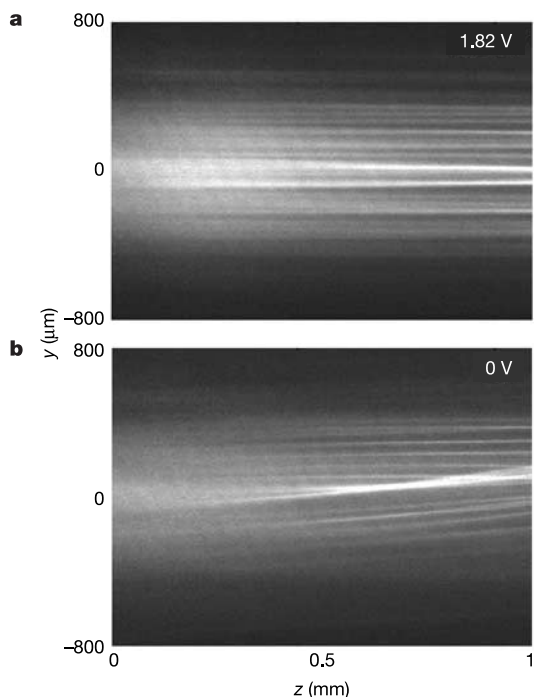


Figure 4 Observation of anisotropic modulational instability and generation of multiple spatial solitons for two different biases. **a**, $V = 1.82$ V; **b**, $V = 0$ V, corresponding to apparent walk-off angles $\alpha \approx 1.5^\circ$ and $\alpha \approx 7^\circ$, respectively.

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Unusual phase transitions in ferroelectric nanodisks and nanorods

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Bulk ferroelectrics undergo structural phase transformations at low temperatures, giving multi-stable (that is, multiple-minimum) degenerate states with spontaneous polarization. Accessing these states by applying, and varying the direction of, an external electric field is a key principle for the operation of devices such as non-volatile ferroelectric random access memories¹ (NFERAMs). Compared with bulk ferroelectrics, low-dimensional finite ferroelectric structures promise to increase the storage density of NFERAMs 10,000-fold². But this anticipated benefit hinges on whether phase transitions and multi-stable states still exist in low-dimensional structures. Previous studies have suggested that phase transitions are impossible in one-dimensional systems^{3–5}, and become increasingly less likely as dimensionality further decreases^{3–6}. Here we perform *ab initio* studies of ferroelectric nanoscale disks and rods of technologically important Pb(Zr,Ti)O₃ solid solutions, and demonstrate the existence of previously unknown phase transitions in zero-dimensional ferroelectric nanoparticles. The minimum diameter of the disks that display low-temperature structural bistability is determined to be 3.2 nm, enabling an ultimate NFERAM density of 60×10^{12} bits per square inch—that is, five orders of magnitude larger than those currently available⁷. Our results suggest an innovative use of ferroelectric nanostructures for data storage, and are of fundamental value for the theory of phase transition in systems of low dimensionality.

One main difference between ferroelectric (FE) nanostructures and (infinite) bulk materials is the existence in the former of depolarizing field, because of the uncompensated charges at the surface⁸. The depolarizing field (the magnitude of which can be as high as 10^4 kV cm⁻¹) is able to quench spontaneous polarization; this is consistent with the recent experimental and theoretical findings that the ground states of finite free-standing FE samples remain paraelectric at very low temperature (50 K)^{9,10}. To induce sizable polarizations, external electric fields^{9,11}—or equivalently, as pointed out in ref. 12, short-circuit boundary conditions^{13,14}—are needed to screen the depolarizing field. As the external fields vanish, the induced polarizations are expected to relax to the ground state of non-polarity, with the relaxation time decreasing exponentially with reducing size, which hampers the miniaturization of nanoscale NFERAMs. Further, a large external electric field is needed to reverse the polarization and to swap charges in electrodes in order to rewrite data bits.

Whether phase transitions still occur in low-dimensional structures has been a subject of long-standing fundamental interest for understanding and revealing collective interactions^{3–6}. For instance, ref. 3 predicts that the thermal fluctuation of acoustic phonons and the entropy due to domain formation disfavour long-range ordering of local dipoles in one-dimensional systems, without

any transition to phases with spontaneous polarization. Similarly, using a spin-lattice model, Mermin and Wagner showed that no spontaneous magnetic ordering exists in one-dimensional systems⁴.

Here we report state-of-the-art *ab initio* simulations, which lead to the discovery that (1) phase transitions do in fact exist in zero-dimensional FE nanostructures; (2) these phase transitions differ profoundly from those occurring in bulk material, in the sense that they lead to the formation of spontaneous toroid moment¹⁵ rather than spontaneous polarization, below a critical temperature; (3) the unusual characteristics of the resulting low-temperature phases promise the generation of new NFERAM devices with remarkable capabilities. Our simulations also reveal the precise role of finite-size effects on toroid moments.

We study free-standing nanoparticles of perovskite Pb(Zr_{0.5}Ti_{0.5})O₃ (PZT) solid solution—the most promising candidate for nano-NFERAM and nano-MEMS^{2,16}. The investigated nanoparticles all have a cylindrical shape, with diameter D and height H (both in units of bulk lattice constant $a = 4$ Å throughout this paper); the cylindrical z axis is chosen to be along the pseudocubic [001] direction, with the x and y axes along the [100] and [010] directions, respectively. Particles with $D > H$ and $D < H$ are referred to as disks and rods, respectively, and each particle is named as (D,H) . A variety of combinations of D and H , ranging from 5 to 30, are considered here.

Technically, a first-principles-derived effective hamiltonian^{17,18} is used to determine the energetics and local dipoles in each perovskite five-atom cell. (This hamiltonian has been shown to reproduce well the observed thermodynamic behaviour of bulk PZT, including the occurrence of an unusual monoclinic phase for a small range of Ti composition^{18,19}.) Nanoparticles surrounded by vacuum are mimicked without periodic boundary conditions; details of our approach for FE nanoparticles are described elsewhere¹⁰. The validity of this approach was demonstrated by the accurate determination of the poling fields in BaTiO₃ dots¹⁰, as well as by the theoretical study of ultrathin PZT films under compressive strains that yield a 180° stripe domain²⁰, in agreement with experimental observation²¹. Here we focus on atomically disordered PZT nanostructures, which are consistent with the chemical nature of bulk PZT²².

For all simulated nanoparticles, the total net polarization $\mathbf{P} = N^{-1} \sum_i \mathbf{p}_i$ (where $\mathbf{p}_i$ is the local dipole of cell i located at $\mathbf{R}_i$, and N is the number of cells in the simulation) is found to be null down to 10 K. Unlike previous studies that mainly focused on spontaneous polarization as the evidence of phase transition, we, on the other hand, discover a new order parameter—namely, the toroid moment $\mathbf{G}$ of polarization—defined as

$$\mathbf{G} = (2N)^{-1} \sum_i \mathbf{R}_i \times \mathbf{p}_i \quad (1)$$

Figure 1a shows that the z component of toroid moment, G_z , of the (19,14) disk increases sharply below 600 K while being zero at higher temperature (the G_x and G_y components remain nearly null at all temperatures). This indicates that an ordering associated with local dipoles occurs in this zero-dimensional disk below a certain critical temperature, T_c . We further found that the specific heat (not shown here) exhibits a hump around 550 K, which provides a quantitative measure of T_c and further confirms the existence of a phase transition.

In order to gain a microscopic insight into this unusual phase transition, Fig. 1b shows the contribution of each (001) plane in the (19,14) disk to the total G_z at high (768 K) and low (64 K) temperature. One can clearly see that the moments of individual planes are all small and random at 768 K. On the other hand, these moments markedly increase in magnitude, and also spontaneously order along the z direction, when the temperature is lowered. These low-temperature local toroid moments are predicted to be nearly identical in each (001) plane, except near the surface layers. The resulting ordered phase, which we denote as phase A, is character-

ized by either clockwise or anti-clockwise vortices in each z plane, with a vortex in the central z plane displayed in the inset of Fig. 1b.

We now explore how this peculiar A phase, and its characteristics, evolve as a function of diameter D for a fixed height H (chosen here to be 14). The most notable results are that: (1) the A phase is found to be stable (at low temperature) 'only' above a critical value of D —denoted by $D_{c,A}$, and equal to 8 when $H = 14$; (2) low-temperature toroid moment G_z increases in magnitude as the diameter becomes larger, with an inflexion point occurring in the G_z - D curve when meeting the $D = H$ equality—that is, when nanorods become nanodisks (see Fig. 2a); (3) the T_c of nanorods ($D < H$) markedly increases as D increases, whereas nanodisks ($D > H$) exhibit a diameter-insensitive T_c (see Fig. 2b).

Result (1), indicated above, raises the question of what structurally happens at low temperature in FE nanoparticles with diameter smaller than $D_{c,A}$. We numerically found that below a second critical diameter (to be referred to as $D_{c,B}$, and equal to 6 when $H = 14$), no vortices exist in any plane, yielding a vanishing total toroid moment. On the other hand, substantial off-centre displacements still occur, with the spontaneous polarization being still null. The resulting macroscopically 'non-polar' and 'non-toroidal' phase can be characterized as spin-glass type²³, and will be denoted as the SG phase. Another feature that we discover is that the A phase does not directly transform into this glass-type SG phase, when decreasing the diameter. In fact, when D ranges between $D_{c,A}$ and $D_{c,B}$, a new structural phase—to be referred to as the B phase—forms. The arrangement of the local dipoles in this B phase is depicted in Fig. 1c, for the (7,28) nanorod and at low temperature.

Like the A phase and unlike the SG phase, phase B exhibits vortices with non-zero local toroid moments. However, unlike the A phase, the toroid moments of phase B have x and y components but no z component. Specifically, Fig. 1c shows that, in the (7,28)

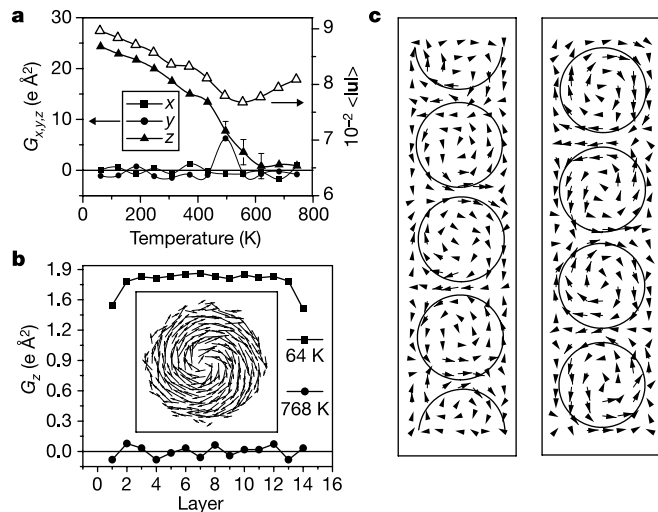


Figure 1 Toroid moment $\mathbf{G}$ and local dipole pattern in PZT disks and rods. **a**, $G_{x,y,z}$ (filled symbols, using the left axis) and average amplitude of off-centre displacement $\langle |\mathbf{u}| \rangle$ (in units of bulk lattice constant a , open triangles, using the right axis) as a function of temperature for the (19,14) disk. Temperature is scaled so that the theoretical Curie temperature for bulk $\text{Pb}(\text{Zr}_{0.5}\text{Ti}_{0.5})\text{O}_3$ matches the experimental value (640 K). Our simulated $\langle |\mathbf{u}| \rangle$ at 750 K is found to be $\sim 0.08a$, which is 75% of the corresponding value at 32 K, in good agreement with the experimental NMR results in bulk PbTiO_3 (ref. 29) where the local distortion at $T_c + 190$ K (being $0.077a$ for Ti) is 70% of its value at 12 K. For G_z near the critical temperature, statistical uncertainty (obtained from averaging over different numbers of Monte Carlo steps in simulation) is indicated by error bars. A sizable G_y at 500 K is due to statistical fluctuation. **b**, Contributions to the total G_z from each (001) plane in the (19,14) disk at 768 K and 64 K. Inset: local dipoles on the central $z=7$ plane, at 64 K (the magnitude of each dipole is enlarged for clarity). **c**, Local dipoles on the central x (the left panel) and y cross-section (the right panel) of the (7,28) rod at 64 K. Local vortices are schematically shown as circles.

nanorod, four vortices appear in the central x (as well as y) cross-section—and intriguingly, each vortex is found to have a diameter nearly the size of D , with two neighbouring vortices having opposite local toroid moments. The total toroid moment, unlike the local toroid moments, is thus relatively small in the B phase. This B phase can be thought of as an intermediate phase between the A phase (characterized by large local and total toroid moments) and the SG phase (for which there is neither local nor total toroid moment), occurring via the quasi-annihilation of the total, but not local, toroid moment. Furthermore, Fig. 1c shows that the edges of the vortices on the x plane go through the centres of the vortices in the y plane: two sets of vortices in the B phase are therefore interconnected like links in a chain. As a result, the local toroid moments adopt a helix-like ordering with a period $\lambda \approx 2D$, and this B phase is 16-fold degenerate.

Figure 2a shows the evolution of the magnitude of total toroid moment $G_{xy} = (G_x^2 + G_y^2)^{1/2}$ in the B phase, as a function of the rod

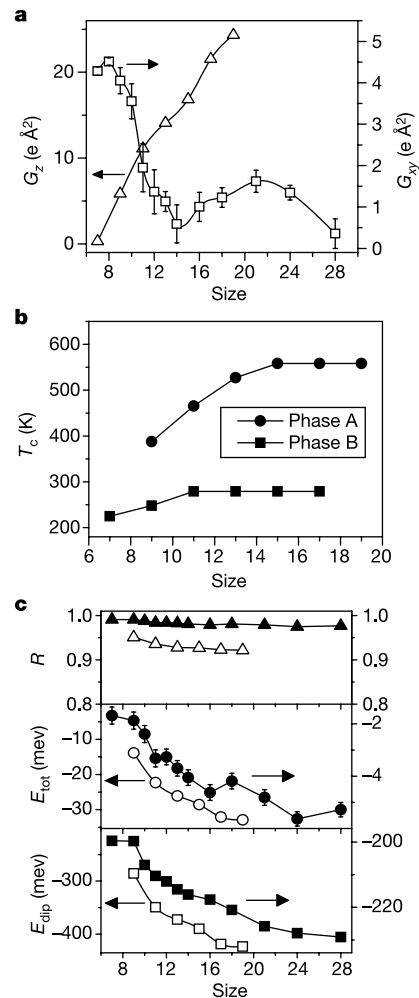


Figure 2 Size dependence of properties of the A and B phases. **a**, Toroid moment G_z of phase A (triangles, using the left axis) and G_{xy} of phase B (squares, using the right axis), at 64 K. **b**, Critical temperatures for the A and B phases. Notably, the T_c of the A phase (being larger than 350 K) indicates that the toroid moments in PZT nanoparticles are conveniently ready for making a new generation of NFERAMs able to operate at room temperature. **c**, Dipole energy E_{dip} , total energy E_{tot} per five-atom cell, and ratio $R = [E_{n-\text{dip}}/E_{\text{dip}}]$, at 64 K. Open symbols, A phase (using the left axis); filled symbols, B phase (using the right axis). The size on the horizontal axis in each figure is the diameter D for phase A (while H is fixed at 14), or height H for phase B (while D is fixed at 7). The error bars indicate the statistical uncertainty in our simulations. For those results with less than $\pm 3\%$ uncertainty, error bars are not shown for clarity.

height at low temperature. We can see that this magnitude is relatively small with respect to the G_z of the A phase, and decreases non-monotonically as H increases. Moreover, Fig. 2b shows that the critical temperature at which the B phase forms is considerably lower than the T_c at which the A phase appears.

The local dipoles in ordered A phase bear a remarkable resemblance to the molecule orientations in the so-called smectic phase of liquid crystals²⁴—and the ordered dipoles in B phase resemble orientations in the cholesteric phase. The above FE patterns in A or B phases are found to be robust, in the sense that they do not depend significantly on the surface termination of nanoparticles, or on whether the short-range interaction between vacuum and the local modes beyond the first surface layer is included or not in the simulations, since the patterns are predominantly determined by the long-range electrostatic interaction.

To understand the stabilities of phases A and B, the total energy E_{tot} and dipole energy E_{dip} were determined, and are shown in Fig. 2c at a fixed and low (64 K) temperature. We find that the delicate balance between non-dipolar and dipolar interactions (such balance is believed to play a critical role in affecting the collective behaviour of ferroelectrics²⁵, and is here described quantitatively by the ratio $R = |E_{\text{n-dip}}/E_{\text{dip}}|$, where $E_{\text{n-dip}} = E_{\text{tot}} - E_{\text{dip}}$) is, surprisingly, mostly size-independent—being 0.92 and 0.97 for the A and B phases, respectively. However, the stability energy E_{tot} progressively decreases in magnitude when D is reduced in phase A, which explains the size-dependence of the critical temperature (for the A phase to appear) in Fig. 2b. Furthermore, $|E_{\text{tot}}|$ of phase B (~ 4 meV) is substantially smaller than its counterpart in phase A, hence leading to much lower critical temperatures.

The occurrence of phase transitions in zero-dimensional nanoparticles, in contrast with the predictions for one-dimensional systems^{3,4}, has a rather simple explanation. FE domains prevent phase transitions from occurring in one-dimensional systems because they are able to lower the Helmholtz free energy $F = E_{\text{tot}} - TS$ by increasing the entropy S . On the other hand, these domains become energetically unfavourable in zero-dimensional nanostructures (therefore allowing the existence of phase transitions) because the typical size d_{PD} of three-dimensionally confined zero-dimensional domains (denoted as particle domains) is of the order of 100 nm according to experimental measurement²⁶ and thus substantially larger than the size of the studied nanoparticles. We point out that d_{PD} is much larger than the size of planar domains in bulk materials²⁷, because the small surface-to-bulk ratio in the latter case decreases the strain energy at the domain interface and thus allows narrower domains. Also, d_{PD} is significantly different (as it should be) from the small size (~ 2 nm) of stripe domains found in compressed ultrathin films^{20,21} where the substrate-enhanced out-of-plane polarization causes the attractive electrostatic interaction between different domains to become dominating, and in contrast, the strain energy at the domain interface due to short-range bond distortion plays only a minor role (which explains why the domain size can be small). Theoretical determination of d_{PD} is hindered by the limits of computing facilities. In either A or B phase, the local dipoles are ordered in a specific way that minimizes the depolarizing field, while simultaneously forming toroid moments, as in some magnetic systems²⁸. A possible alternative way to eliminate the depolarizing field is to form 180° domains with polarization in each region pointing along the $+z$ or $-z$ direction. This configuration is found, however, to be less stable by our constrained simulation (in which only the z -component of each dipole is non-zero and allowed to relax): the energy per five-atom cell in the (19,14) disk of such 180° domains is determined to be -11 meV, much higher than that of the ground state with toroid moment (-32 meV).

We now consider if the existence of a macroscopic toroid moment, as in phase A—rather than a spontaneous polarization, as in bulk ferroelectrics—could lead to the design of new or improved technological devices. Here it is important to realize

that phase A is bistable (that is, the toroid moment can be equivalently parallel or anti-parallel to the z -axis). Unlike the situation in bulk ferroelectrics where states with differently oriented polarization can be accessed via a static external electric field, we can switch from one minimum of phase A to the other by applying a time-dependent magnetic field. This magnetic field, generating a curling electric field via $\nabla \times \mathbf{E} = -\partial \mathbf{B}/\partial t$, interacts with the total toroid moment of the PZT particles, as described by the energetic term $E_{\text{int}} = -(2N)^{-1} \sum_i \mathbf{p}_i \cdot [\mathbf{R}_i \times (\nabla \times \mathbf{E})] = \mathbf{G} \cdot \partial \mathbf{B}/\partial t$. The coercive field $\partial \mathbf{B}/\partial t$ needed to switch the toroid moment of the (19,14) disk is estimated from the total energy E_{tot} to be $1.6 \text{ mV \AA}^{-2}$ (the real coercive field may be different because of nucleation and tunnelling).

Storing data using a switchable macroscopic toroid moment could be superior to using spontaneous polarization, because generating a magnetic field—unlike the generation of electric field—does not require electrode contact, which is challenging to make in nanoscale devices. Furthermore, a large number of particles have to be arranged into regular arrays for memory nanodevices to be efficient, but they should not interact strongly (in order to avoid the so-called ‘cross-talk’ problem). Phase A does not exhibit any macroscopic polarization, or produce a strong electric field that has a long-range character. The vortex structure of phase A in a single nanoparticle can therefore be switched without modifying the states of its neighbouring particles. Consequently, the toroid carriers of information can thus be packed considerably more densely than the conventional carriers of polarization, giving rise to a marked improvement in the density of ferroelectric recording. For instance, the minimum diameter that we found to be able to generate bistable toroid states is 3.2 nm. This produces an ultrahigh storage density of $60 \text{ Tbit inch}^{-2}$, which is five orders of magnitude larger than current NFERAM capability⁷ of $0.2 \text{ Gbit inch}^{-2}$. Such a promising capability also far surpasses the 1 Gbit inch^{-2} density of typical magnetic recording.

We also examined the electric field generated by the toroid phase in the (19,14) disk, and found that this field is measurable in the proximity of the nanodisk, though it decays away quickly (thus implying that virtually no cross-talk occurs). Furthermore, the electric-field pattern (namely, the spatial dependence of the field magnitude and direction) generated by the toroidally ordered dipoles is found to be very different from that generated by aligned local polarizations. Measuring the electrostatic field using atomic-force-microscope tips may thus confirm the existence of toroid moments and read the memory bits. The toroidally ordered structure may also be revealed by studying the fine structure of synchrotron X-ray diffraction of nanodisk arrays²¹.

We thus hope that our results will not only lead to more interest in the fundamental properties of phase transitions in ferroelectrics, but will also initiate an innovative approach to using ferroelectric nanostructures in devices with unprecedented performance. □

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Wet periods in northeastern Brazil over the past 210 kyr linked to distant climate anomalies

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The tropics are the main source of the atmosphere's sensible and latent heat, and water vapour, and are therefore important for reconstructions of past climate¹. But long, accurately dated records of southern tropical palaeoclimate, which would allow the establishment of climatic connections to distant regions, have not been available. Here we present a 210,000-year (210-kyr) record of wet periods in tropical northeastern Brazil—a region that is currently semi-arid. The record is obtained from speleothems and travertine deposits that are accurately dated using the U/Th method. We find wet periods that are synchronous with

periods of weak East Asian summer monsoons², cold periods in Greenland³, Heinrich events in the North Atlantic⁴ and periods of decreased river runoff to the Cariaco basin⁵. We infer that the wet periods may be explained with a southward displacement of the Intertropical Convergence Zone. This widespread synchronicity of climate anomalies suggests a relatively rapid global reorganization of the ocean–atmosphere system. We conclude that the wet periods probably affected rainforest distribution, as plant fossils show that forest expansion occurred during these intermittent wet intervals, and opened a forest corridor^{6–8} between the Amazonian and Atlantic rainforests.

The study area is in northern Bahia state (10° 10' S, 40° 50' W; 500 m above sea level), the centre of the drought-prone semi-arid region of northeastern Brazil, 500 km west of the tropical Atlantic (Fig. 1). Mean annual temperature is 28 °C with seasonal variations <2 °C. Average annual rainfall is ~490 mm with high seasonality and a net moisture deficit caused by high potential evapotranspiration rates (>1,400 mm). Modern vegetation comprises shrub-like drought-resistant caatinga. The area is characterized by a well-developed underground karst containing some of the longest caves in the Southern Hemisphere, such as the 105-km-long Toca da Boa Vista (TBV).

Calcite speleothems were collected from TBV, Lapa dos Brejões (LBR) and Toca da Barriguda (TBR) caves (see Methods), and travertine deposits were collected from the surrounding Salitre and Jacaré river valleys. As in semi-arid southeastern Australia⁹, low rainfall and high evapotranspiration in northeastern Brazil preclude speleothem and travertine deposition during dry intervals such as today. Therefore, speleothem and travertine formation unequivocally indicates wetter conditions in the past. Travertine surface deposits may be generated by relatively small increases in rainfall, as rainfall directly feeds bicarbonate-rich surface runoff. For speleothems to precipitate, however, soil and bedrock infiltration thresholds must be overcome; hence speleothem growth phases probably correspond to high rainfall periods. Wetter climates in the past were suggested by geological and geomorphological evidence from northeastern Brazil¹⁰ and ocean sediment records off the continental margin of northeastern Brazil¹¹. However, the chronological resolution of the data limited detailed correlation with climate elsewhere. Here, we further constrain the timing of past pluvial phases in northeastern Brazil by systematic sampling and high-precision mass spectrometric ²³⁰Th dating of growth phases of multi- and single-phase speleothems and travertines (see Methods).

Fifty-four ²³⁰Th ages from 11 speleothems and 55 U/Th analyses on travertines (Supplementary Tables S1 and S2, Supplementary Fig. S1) were obtained with thermal ionization¹² and inductively coupled plasma¹³ mass spectroscopic techniques (see Methods). Most speleothem ages have 2σ analytical errors that correspond to ~0.5–1% of the age. Ages are in correct stratigraphic order within quoted uncertainties and corrections for initial ²³⁰Th are negligible.

Speleothem growth in northeastern Brazil was highly episodic. Growth phases were as short as several hundred years, with some lasting up to a few thousand years, typically separated in time by several thousands to several tens of thousands of years (Fig. 2), indicating extended intervals of dry conditions punctuated by short pluvial periods. Overall, periods of speleothem growth occupied only a small fraction (~8%) of the last 210 kyr, indicating that pluvial conditions were uncommon in the area.

Because of the high precision of our ages, we are able to correlate the pluvial periods to climate events recorded in other high-resolution records. The Hulu cave East Asian monsoon record², which covers most of the last glacial period, has comparable precision. Furthermore, the events at Hulu have been correlated with climate in Greenland³, as have events recorded in the Cariaco basin⁵, and Heinrich events in the North Atlantic⁴. To the extent that these correlations are accurate, we can compare the timing of our

pluvial periods to climate at the other localities, relying on our temporal precision and that of the Hulu record.

Our last glacial period pluvial phases (~15 kyr ago, 39 kyr ago and 48 kyr ago, and six speleothem growth intervals between 60 and 74 kyr ago) correlate precisely with times of particularly high $\delta^{18}\text{O}$ values at Hulu cave², inferred to represent dry conditions associated with a weak summer East Asian monsoon (Fig. 2). The latter in turn correlates with Greenland ice-core cold events³, largely associated with Heinrich events⁴, and with times of high reflectance in Cariaco

basin sediments that represent periods of low river runoff in northern South America⁵. Pluvial periods correlating with Heinrich events H1, H4, H5 and H6 are represented in our growth phases, but neither H2 nor H3, which occur at times of low austral autumn insolation, are represented. Dating of the pluvial phase correlated to H4 is precise enough to resolve its duration (700 ± 400 yr from 39.6 to 38.9 kyr ago, on the basis of top and bottom dates of a growth phase of stalagmite TBV 40, Supplementary Fig. S2).

Before ~75 kyr ago, correlations are more difficult as there are

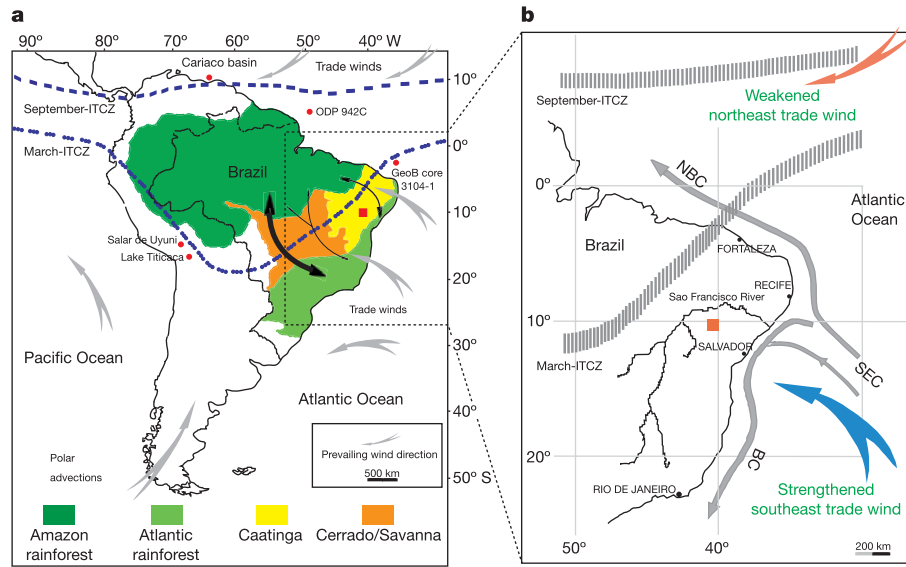


Figure 1 Location of study area, present ITCZ seasonal positions, dominant wind directions, ocean surface currents, and main vegetation distributions in Brazil (modified from ref. 8). Also shown is the Brazilian geographical boundary (black line). **a**, Red square indicates the study area. The 'pathways' connecting the Amazon and the Atlantic rainforest hypothesized by Por⁶ are represented by double-headed arrows. The thickness

of the arrows indicates the degree of past biogeographical connections. **b**, Grey arrows indicate dominant surface and near-surface ocean currents: NBC, North Brazil Current; BC, Brazil Current; SEC, South Equatorial Current. The blue arrow shows the current relatively strong southeast trade wind; the red arrow represents the currently relatively weak northeast trade wind.

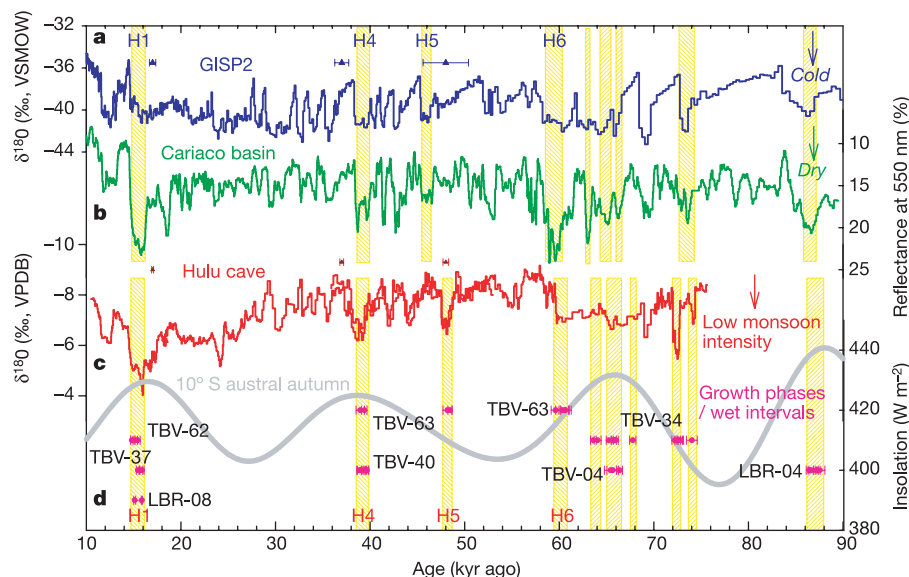


Figure 2 Comparison of the growth patterns of speleothems from northeastern Brazil with events recorded in several Northern Hemisphere palaeoclimate archives. **a**, $\delta^{18}\text{O}$ values of Greenland ice³. **b**, Light colour reflectance (greyscale) of the Cariaco basin sediments from ODP Hole 1002C⁵. **c**, $\delta^{18}\text{O}$ values of Hulu cave stalagmites². **d**, Speleothem growth patterns in northeastern Brazil. Growth intervals are shown by separated pink dots or connections between dots if they are within the same phase. 2σ dating errors (error bars)

are typically 0.5–1%. Yellow vertical bars indicate possible correlations between four records. Also shown are dating errors for the GISP2 ice core²⁹ (blue bars) and Hulu cave speleothems² (red bars), and 10°S austral autumn insolation³⁰ (grey line). VSMOW, Vienna standard mean ocean water. VPDB, Vienna PeeDee Belemnite. H1, H4, H5, H6, Heinrich events.

fewer comparable high-resolution records. Nevertheless, the growth phase between 86.5 ± 0.4 and 87.2 ± 0.3 kyr ago probably correlates with a cold period in Greenland between Greenland interstadials (GIS) 21 and 22³ (Fig. 2), and an associated dry period recorded in the Cariaco basin⁵. Growth phases between ~ 130 and 134 kyr ago (Fig. 3) corresponded to a period of weak Asian monsoon just before an abrupt increase (at 129.3 ± 0.9 kyr ago) in monsoon intensity that marks monsoon termination II¹⁴ and with H11¹⁵. Growth phases from 178 to 182, 205 to 206, and 207 to 209 kyr ago correlate broadly with events within marine isotope stages (MIS) 6.6 and 7.2¹⁶.

The travertine record extends and complements the speleothem record because it probably includes periods of growth at times not wet enough to promote speleothem growth. Represented in the travertine (Fig. 3), but not the speleothem, record are times within the Younger Dryas (ages between 11.7 and 12.1 kyr ago) and the Last Glacial Maximum (ages between 16.7 and 21.7 kyr ago). Some ages fall between 350 and 450 kyr ago. A large number of sub-samples have ages greater than the ^{230}Th dating limit (Supplementary Fig. S1); on the basis of their measured $\delta^{234}\text{U}$ values (~ 450) and the initial $\delta^{234}\text{U}$ values ($\sim 6,000$) of neighbouring younger travertines, the ages of these old travertines are about 0.9 to 1.0 million years, indicating pluvial periods as old as the mid-Pleistocene.

Because modern rainfall to the north is largely associated with the Intertropical Convergence Zone (ITCZ)¹, our pluvial periods probably represent times when the southerly limit of the ITCZ was in (or south of) our field area, more than several hundred kilometres south of its present southern limit. If so, a southward shift in the southern limit of the ITCZ occurs when Greenland air temperature is low, the East Asian monsoon is weak, and the northern limit of the ITCZ is to the south. As the monsoon front is related to the ITCZ¹⁷, these observations can be summarized in terms of a southward shift of various manifestations and geographical limits of the ITCZ, at times of cold temperature in Greenland.

In recent decades, the Atlantic 'meridional mode' may have played a role in controlling the position of the Atlantic portion of the ITCZ¹⁸. Dry anomalies in northeastern Brazil correlate with negative sea surface temperature (SST) anomalies in the southern tropical Atlantic and positive anomalies in the northern tropical

Atlantic^{19,20}. This SST pattern strengthens the southeast trade winds, displacing the ITCZ to the north (Fig. 1). Conversely, temperature anomalies in the opposite sense strengthen the northeast trade winds, causing a southward shift in the ITCZ, as observed in our study.

Our data confirm model predictions that southward expansion of Northern Hemisphere ice sheets and northern sea-ice cover caused a southward shift in the ITCZ position—in part, by establishing meridional mode SST patterns²¹. It is also possible that millennial-scale changes in ocean heat circulation related to the bipolar see-saw mechanism²² could change meridional Atlantic SST gradients, shifting the ITCZ. In a broader sense, it has been argued that the position of the ITCZ is controlled by the relative pole-to-Equator temperature gradients between hemispheres, with the ITCZ offset to the hemisphere with the lower temperature gradient²³. If cold Greenland temperatures correspond to times of high Northern Hemisphere latitudinal temperature gradient, this would explain the broad link between ITCZ position and Greenland temperature.

Feedbacks between the tropics and high northern latitude climate may also be important in establishing the observed climate patterns. For example, southward displacement of the ITCZ may change the flow pattern and intensity of the North Brazil Current (NBC)¹¹, which could directly affect heat and salt transport into the Caribbean and ultimately to portions of the North Atlantic. Alternatively⁵, the ITCZ position could modulate water vapour export from the Atlantic to the Pacific. Such changes could also shift Caribbean salinity and affect northward heat transport. Another possible feedback related to ITCZ position could be changes in low-to-high latitude moisture transport associated with changes in the Asian monsoon¹⁴.

Furthermore, over the period 10–210 kyr ago there is a striking correlation between speleothem growth phases and times of high insolation at 10°S during austral autumn (Fig. 3). Modern rainfall in this area occurs mainly during austral autumn (February to May)¹, when the ITCZ is close to its southernmost position. Our data thus confirm the strong dependency of the ITCZ position and associated tropical precipitation on precessional forcing demonstrated in modelling²⁴ and field studies in South America²⁵. Increased continental heating and land/sea thermal contrast could

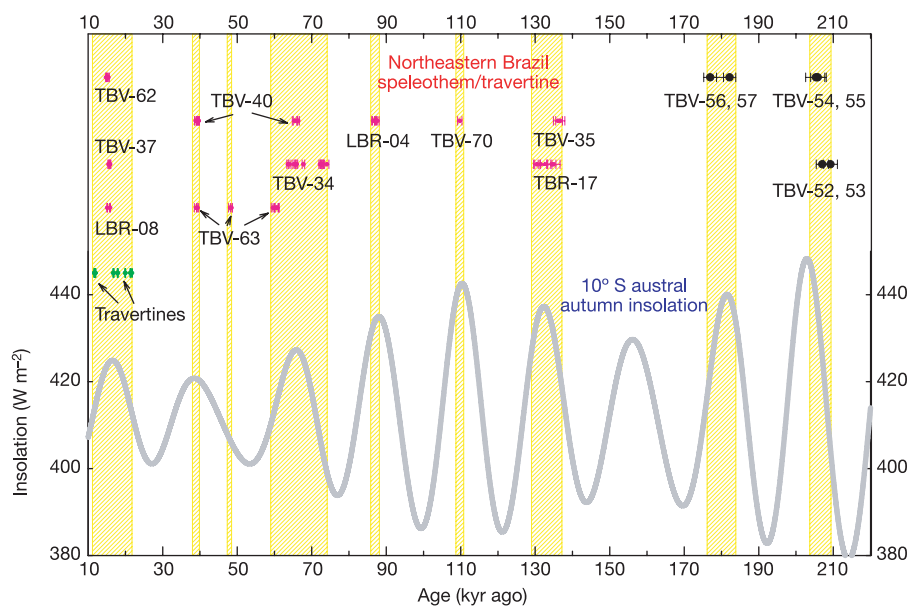


Figure 3 Comparison of growth intervals of speleothem and young travertine from northeastern Brazil, and insolation variation at 10°S over the austral autumn³⁰. Pink, black and green dots represent respectively stalagmite, flowstone (a type of speleothem) and travertine.

shift the ITCZ to the south and enhance landward water vapour transport^{1,25}.

The patterns of latitudinal migration of the ITCZ and rainfall inferred here imply geographical shifts in rainforest boundaries that, in turn, influenced rainforest biodiversity and evolution. Analysis of widespread vegetal remains associated with the travertines (Supplementary Fig. S3) has allowed the identification of plant species not adapted to the present semi-arid climate, with less than 5% coriaceous leaves (predominant in modern caatinga vegetation). Many specimens show brochidodromous venation, common in the modern Atlantic rainforest but not in the caatinga. The travertine palaeobotanical remains indicate a dense mesophilic semi-deciduous forest that could have replaced, or mixed with, the caatinga. The wide occurrence of vegetation-rich fossil travertines in northeastern Brazil indicates that the expansion of forest was a regional phenomenon during wet phases.

Historically, the existence of 'refugia' was proposed as a critical factor in development of tropical rainforest biodiversity²⁶. Central to this idea is the large-scale drying of the tropical forest lowlands during glacial times, leaving 'refuges' of rainforests at wetter higher elevations. However, some pollen studies suggest that Amazonian lowland vegetation may not have changed significantly during the late Pleistocene²⁷. An alternative explanation supported by botanical and genetic studies^{6–8,28} is that the high biodiversity resulted from periodic exchanges between the Amazonian and Atlantic rainforests. Our data show that the currently semi-arid southeastern border of the Amazonian rainforest experienced relatively frequent, dramatic and abrupt changes in available moisture during the Pleistocene. These intermittent wetter conditions allowed the expansion and extension of gallery forest, and floristic exchange between the two forest areas. The precise timing, frequency and mechanism for such connections were previously unknown. Our data indicate that relatively frequent intermittent connection (on average one pluvial event every ~20,000 yr for the past 210 kyr) through ITCZ migration has been possible since at least the mid Pleistocene, a process which probably affected rainforest species dynamics and biodiversity. □

Methods

Sample description

Calcite speleothems were collected from deep caves in the Precambrian Una carbonates, northeastern Brazil. Conservation ethics (and permit restrictions) limited sample collection, and only 11 speleothems could be analysed. We systematically sampled the oldest and youngest suitable calcite from each sample, and also collected sub-samples bracketing hiatuses to constrain speleothem growth intervals by high-precision mass spectrometric ²³⁰Th dating (see Supplementary Fig. S2 for an example). Some duplicates and sub-samples in the middle of growth phases were also collected to check analytical precision and growth rate.

Travertine deposits were collected from the surrounding Salitre and Jacaré river valleys. Travertine sites are underlain by the thin impermeable Caatinga limestone. Rapid rainfall infiltration towards the limestone causes seepage to concentrate in shallow streams that cascade steeply into the main river valley, causing travertine deposition via degassing and/or evaporation. It was necessary to establish strict age reliability criteria because of the friable and porous nature of travertines. Two main criteria were adopted for travertine sample selection: (1) coeval sub-samples (that is, different samples from the same growth layer) were analysed for all samples. Because post-depositional isotopic migration is unlikely to affect different portions of the same layer in exactly the same way, a comparison of isotopic ratios and age provided a reliable check on age accuracy. (2) Top and bottom sub-samples were analysed whenever possible in order to check for stratigraphic order. Some larger travertine outcrops contain shallow caves. Calcite deposits within these caves were sheltered from weathering and mixing with terrigenous sediment, and tended to yield calcite samples that were free of impurities and less likely to be altered (see Supplementary Table S2).

U-series experimental methods

Speleothem sub-samples (~0.1–0.3 g) for dating were extracted by milling from flat, polished surfaces using a hand-held dental drill. For travertines, small pieces (0.1–0.4 g) were cleaned using an ultrasonic cleaner and rinsed with deionized water to remove surface contamination. Procedures for chemical separation and purification of uranium and thorium are similar to those described in ref. 12. Some travertine samples carried a HNO₃-insoluble residue. In these cases, we completely dissolved the detritus with a concentrated HF–HClO₄ mixture.

A few U and Th measurements were made at the Minnesota Isotope Laboratory by

thermal ionization mass spectrometry. Following instrumental procedures similar to those of ref. 12, measurements were made separately on two Finnigan MAT-262-RPQ instruments, using the multiplier behind the retarding potential quadrupole. The majority of the measurements were made by magnet-sector inductively coupled plasma mass spectrometry, following procedures modified from ref. 13. Measurements were performed on a Finnigan ELEMENT, equipped with a double-focusing sector magnet in reversed Nier-Johnson geometry using a single MasCom multiplier.

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Spreading-rate dependence of melt extraction at mid-ocean ridges from mantle seismic refraction data

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A variety of observations indicate that mid-ocean ridges produce less crust at spreading rates below 20 mm yr^{-1} (refs 1–3), reflecting changes in fundamental ridge processes with decreasing spreading rate. The nature of these changes, however, remains uncertain, with end-member explanations being decreasing shallow melting³ or incomplete melt extraction², each due to the influence of a thicker thermal lid. Here we present results of a seismic refraction experiment designed to study mid-ocean ridge processes by imaging residual mantle structure. Our results reveal an abrupt lateral change in bulk mantle seismic properties associated with a change from slow to ultraslow palaeo-spreading rate. Changes in mantle velocity gradient, basement topography and crustal thickness all correlate with this spreading-rate change. These observations can be explained by variations in melt extraction at the ridge, with a gabbroic phase preferentially retained in the mantle at slower spreading rates. The estimated volume of retained melt balances the $\sim 1.5\text{-km}$ difference in crustal thickness, suggesting that changes in spreading rate affect melt-extraction processes rather than total melting.

The processes of lithospheric formation at mid-ocean ridges (MORs)—mantle upwelling and corner flow, decompression melting, melt extraction, crustal accretion—are central to our understanding of mantle dynamics. The shallow upper mantle preserves structure inherited from and diagnostic of these processes, such as the patterns of melt depletion⁴ and shear deformation⁵. These characteristics affect the mantle's seismic structure (for example, velocity and anisotropy). Imaging this structure can thus provide us with knowledge of the processes that have formed oceanic lithosphere at different times and under a variety of conditions.

The main FAIM (far-offset active-source imaging of the mantle) experiment transect, line 1, extends 800 km along a plate-kinematic flow line in the western mid-Atlantic, within a single spreading segment across lithosphere ranging in age from 108 to 157 million years (Myr) ago (ref. 6) (Fig. 1). Slow palaeo-spreading rates here enable us to investigate upper-mantle structure over a 35-Myr age span, and variations in palaeo-spreading rates during this time provide a means of evaluating the rate dependence of MOR processes. Eight pairs of vertical-component, 2-Hz ocean-bottom seismometers (OBSs) were deployed along line 1, spaced 80–120 km apart, and two OBSs were deployed at the ends of the orthogonal line 2, 150 km apart.

A primary result of the FAIM experiment is that mantle refractions sourced by airguns can be recorded at very far offsets (Fig. 2). On line 1, strong mantle refractions are observed to maximum offsets of 375 km by instruments on the eastern half of the transect. This phase is also observed to the maximum offset by both instruments on line 2. However, mantle refractions are not observed by instruments located on the western half of line 1. The lack of observed mantle refractions in the west most probably reflects a change in the propagation properties of the mantle, because noise

characteristics and instrument performance are the same in the east and west, and crustal phases are well recorded on all instruments. The transition from propagation to non-propagation of mantle refractions occurs near kilometre (km) 300 (eastward from the westernmost instrument), and it is abrupt, as evidenced by the coincident truncation at this location of refractions recorded by the instruments east of km 300 (Fig. 2). Primary mantle refractions from shots fired west of km 300 do not propagate back to the sea floor with recognizable energy. The simplest explanation for this energy dissipation is that the vertical velocity gradient west of km 300 is near zero or negative, consistent with conclusions from an explosive-source seismic study in the same region⁷ (Fig. 1).

Mantle refraction travel times for lines 1 and 2 are well fitted with a mantle velocity structure that is approximately one-dimensional (Fig. 3). Velocity models for both lines require substantial vertical gradients (0.014 and $0.024 \text{ km s}^{-1} \text{ km}^{-1}$), with the difference in average velocity indicating $\sim 3\%$ seismic anisotropy⁸. The velocity profile to 20-km depth is constrained by multiple instruments, but the deeper structure is constrained only by the far-offset Tecate travel times. These travel times are well fitted with a single linear gradient extending to a depth of 39 km, or by a model including a small velocity step near a depth of 30 km with a smaller gradient beneath. We prefer the velocity-step model because preliminary amplitude analysis indicates a weak preference for this model (Supplementary Fig. S7), and the deepest velocity in the single-gradient model is high (8.63 km s^{-1}) relative to expectations for anisotropic, depleted peridotite at this depth⁹.

The steep positive velocity gradient in the east is not easily explained by MOR processes involving passive upwelling and efficient melt extraction. It is not explained by the predicted vertical variation in the extent of melting and associated depletion of a basaltic component. Seismic velocity increases with increasing depletion as mantle rock becomes more olivine- and magnesium-rich¹⁰, but the velocity change is small ($<1\%$) and would produce a negative gradient in most models of decompression melting⁴. A

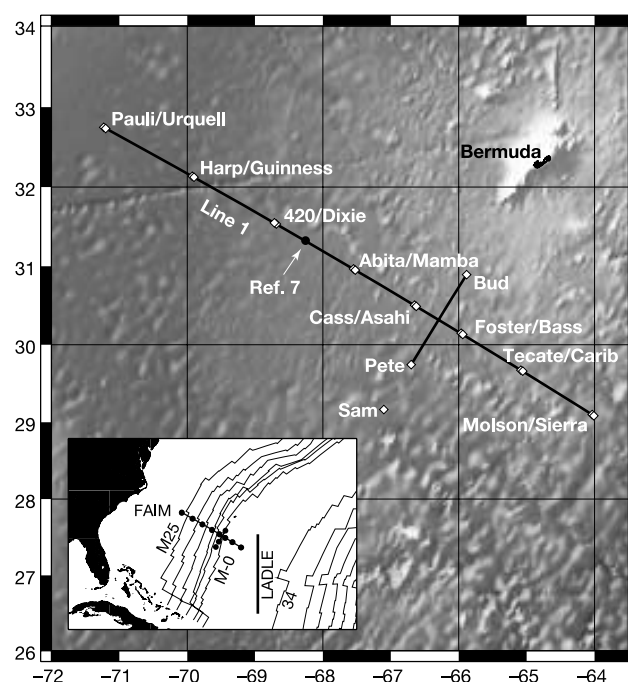


Figure 1 FAIM experiment location and instrument layout. Line 1 lies along a plate-kinematic flow line. The experiment location of ref. 7 is indicated, and the LADLE¹⁹ experiment location and lithospheric isochrons are indicated in the inset. LADLE, Lesser Antilles Deep Lithosphere Experiment.

vertical variation in anisotropy caused by the strain-induced crystal preferred orientation of olivine may yield a positive velocity gradient in one direction, but the gradient in the orthogonal direction should then be nearly flat, or even negative. The gradient is also not explained by the effects of increasing pressure and temperature with depth on elasticity. These effects are small and opposite in sign (Fig. 3), and they should be uniform throughout the region and thus unable to account for lateral transitions in vertical gradient. It is possible that the rougher basement east of km 425 (Fig. 4) indicates a more tectonic mode of extension here, with a more-fractured upper mantle. The closure of cracks with depth can produce important positive velocity gradients, but the predicted effect is too small and shallow to explain the observed gradients (Fig. 3). In the crust, the effect of tectonic cracks on seismic velocity is anisotropic, with a larger effect directed in the palaeo-spreading direction^{11,12}. We would expect a similar anisotropic effect in the mantle, with a strong vertical gradient observed on line 1 and little or no gradient on line 2, but this pattern is not observed.

Our preferred explanation for the observed mantle velocity gradients is motivated by the rough correlation between the apparent velocity-gradient transition near km 300 and changes in basement morphology, crustal thickness and palaeo-spreading rate (Fig. 4). The half-spreading rate decreases from 14 to 13 mm yr⁻¹ at 140 Myr ago (km 325), to 8 mm yr⁻¹ at 132 Myr ago (km 425), and then increases to ~10 mm yr⁻¹ at ~125 Myr ago⁶. (The spreading rate after chron M-0 at km 550 is largely unconstrained. The value in Fig. 4 is an average rate for the entire Cretaceous quiet period, beginning 120 Myr ago.) The large decrease in spreading rate at

132 Myr ago is clearly correlated with roughening of basement topography, suggesting a shift to a more tectonic, less magmatic mode of extension. The crustal thickness measurements indicate that this slower-spreading crust is demonstrably thinner. Melt retained in the mantle at the spreading centre, now present as gabbroic inclusions, thus provides a possible explanation for the change to positive velocity gradients. In this scenario, melt extraction at very slow spreading rates becomes less efficient, owing to the increased effect of conductive cooling. Melt crystallized within the mantle, with the quantity of retained material increasing with decreasing depth, would systematically decrease velocities in proportion with the amount of retained melt. We evaluate this hypothesis with a simple mass-balance calculation. The crust east of km 425 is, on average, 1.4 km thinner than the crust west of km 300. The line-1 velocity gradient to a depth of 30 km can be explained by adding the equivalent of 1.5 km of gabbro (7 km s⁻¹) to mantle rock with an intrinsic velocity of 8.5 km s⁻¹. This simple calculation suggests that a melt-retention explanation for the change in velocity gradient along line 1 is plausible. This suggestion is supported by the fact that the observed gravity anomaly along line 1 is best explained by a lithosphere in isostatic equilibrium with the equivalent of ~1.5 km of crustal-density material distributed within the upper 20–40 km of the mantle (Supplementary Figs S1–S5). The spatial offset (~125 km) between the change in mantle velocity gradient and the change in spreading rate is consistent with the expected off-axis extent of the mantle–melt zone¹³, which also tends to support a melt-retention explanation (Supplementary Fig. S6).

A variety of observations suggest that the retention and crystallization of melt in the mantle is common in slow-spreading environments. The composition of basalts emplaced at slow-spreading ridges suggests that fractional crystallization occurs within the upper mantle to depths of 18 km below the sea floor¹⁴. The composition of dredged plagioclase peridotites¹⁵ is consistent with this notion. Recent drilling along the mid-Atlantic ridge on Ocean Drilling Program Leg 209 recovered gabbroic rocks, found as intrusions and impregnations in residual peridotite, which crystal-

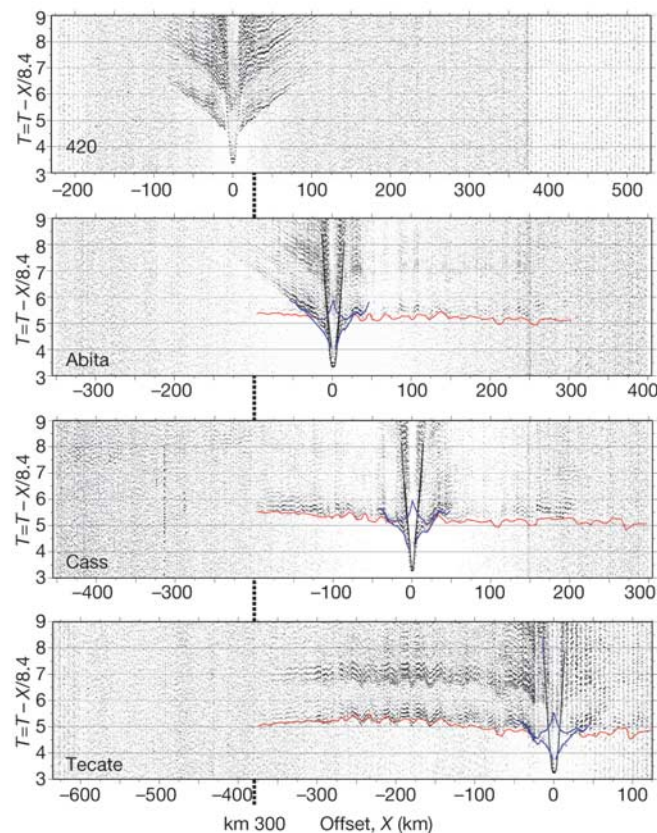


Figure 2 Profiles from FAIM line 1 plotted at reduced travel time T (in seconds), using a reduction velocity V of 8.4 km s⁻¹. Predicted travel times are overlain (black, sea floor and sediments; blue, crustal refractions; red, mantle refractions). The mantle-refraction phase terminates at a distinct location near model km 300. Mantle refractions do not propagate from west of km 300, as seen on instrument 420.

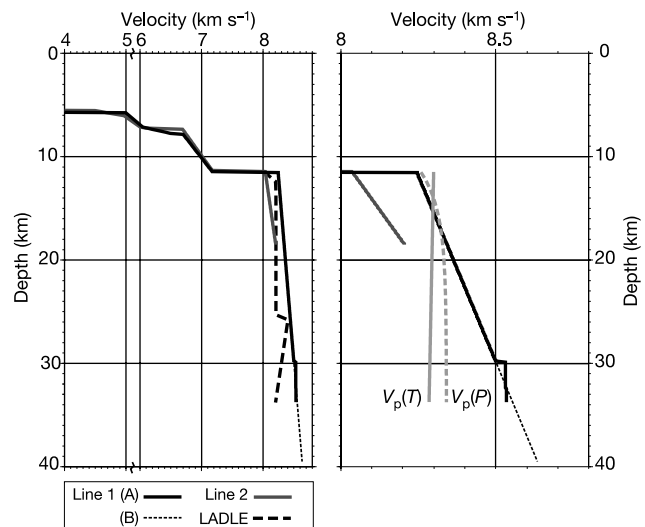


Figure 3 Velocity depth profiles at the line 1/line 2 crossing based on isotropic ray tracing of FAIM travel times. The two line 1 models, A and B, fit the travel-time data equally well, but amplitude analysis favours model B somewhat. The velocity profile based on the LADLE¹⁹ transect, at an azimuth of ~0° (~60° off the flow line), lies between the line 1 and line 2 results. Predicted pressure dependence (based on laboratory measurements on dunite²⁸ and a model of the pressure dependence of P-wave velocity, $V_p(P)$, which isolates the effects of microcracks¹⁰) and temperature³⁰ dependence, $V_p(T)$, are also indicated; the combined effects nearly cancel but predict a slightly negative gradient below ~22 km depth.

lized at depths of up to 17 km beneath the sea floor and are believed to comprise 20–40% of the upper mantle at this location¹⁶.

Our interpretation of the FAIM results suggests that, for slow-spreading systems, spreading rate has a greater effect on melt extraction than on total melt production. This conclusion differs from an interpretation of data from the ultraslow-spreading (half-rate 3–6.5 mm yr⁻¹) Gakkel Ridge, where anomalously thin crust (1.9–3.3 km thick) was taken as evidence for reduced melt production¹⁷. The Gakkel experiment placed some constraints on the uppermost mantle structure, where slow velocities ($V_p \approx 7.5$ – 7.8 km s⁻¹) just below the Moho were interpreted as serpentinized peridotite. An alternative interpretation is that the slow upper-mantle velocities beneath Gakkel are due in part to gabbroic inclusions present in greater proportion to balance the thin crust. Similarly, the systematically enriched rare-earth-element (REE) concentrations observed in basalts from very-slow-spreading ridges have been interpreted to indicate deeper melting with nearly complete melt extraction at these ridges³. Numerical models suggest that this REE enrichment might also be expected in a passively driven system where the off-axis portion of the distributed melt network remains largely trapped, and the crust-forming melts are predominantly extracted from the mantle upwelling directly beneath the ridge axis¹³, the hottest portion of the system (Supplementary Fig. S6).

The presence of a mantle gabbroic component may also provide an explanation for a possible discontinuity near a depth of 30 km, which is similar (~35 km) to the depth where the transformation of olivine tholeiite to eclogite begins, at ~1 GPa (ref. 18). The requirement of this velocity discontinuity is tentative, and further analyses are needed to constrain its existence better. Nevertheless, similar discontinuities have been inferred from other very large offset refraction experiments^{19–22}, with a gabbro-to-eclogite transformation suggested as an explanation. If this explanation is correct, then the apparent common occurrence of such a boundary suggests

that significant quantities of melt may routinely be retained in the mantle under a variety of conditions. □

Methods

Experiment design

Detailed seismic imaging of the uppermost oceanic mantle, to depths comparable to the onset of melting at 60–90 km, can be achieved with active-source techniques employing very large source-to-receiver offsets. A small number of such studies have been carried out in the oceans using explosive sources^{19–22}. These experiments identified large velocity gradients and velocity discontinuities in the upper 50 km of the mantle interpreted as compositional layering. Other than these studies, detailed knowledge of upper-mantle seismic structure away from a ridge is surprisingly poor. This is due in large part to the cost and inherent safety risks of explosive sources. Our experiment was designed to acquire large-offset marine refraction data using airguns rather than explosives. Mantle phases from airgun sources are commonly recorded at 200–300 km offsets by land-based seismometers^{23,24}. Similar observations in the oceans had not been made before the FAIM experiment, however, largely owing to the presence of water-borne ‘previous shot’ noise (PSN)²⁵ endemic at large offsets during airgun shooting with typical shot intervals of 90 s or less. The relationship between shot interval, Δt , and the offset, X_{PSN} , at which a mantle refraction from a particular shot and water-borne energy from the previous shot arrive simultaneously is $\Delta t = X_{PSN}(1/V_W - 1/V_M)$, where V_W and V_M are the water and mantle propagation velocities. To record mantle phases at 600-km offset, for example, without the interference of high-energy PSN, a shot interval of ~6 min is required. The two principal elements of the shooting strategy were thus long shot intervals and repetition of shots in the same (or nearly the same) location to enable signal-to-noise enhancement through stacking. The source for the experiment was a ~150-litre, 20-element airgun array. Two modes of shooting were tested, ‘circle’ shots and ‘on-distance’ shooting. The circle-shooting mode was tested on the eastern 150 km and western 36 km of line 1. Circle shooting involved shooting at a prescribed shot interval while steaming in a circle with radius 1 km, the tightest possible with the towed source array. This is a time-efficient means of generating many shots near a single location. Between 17 and 36 shots were fired along each circle. Static corrections were applied to the traces from each circle, and the traces were stacked. We found that substantial basement roughness and the azimuthal dependence of the source-array signature rendered this mode of shooting problematic. On-distance shooting, where the source is triggered at predefined points by the navigation system, is the simplest and most effective of these two modes; it was used during most of the experiment. A source-point separation of 1 km was used, giving a shot interval of ~6 min. On line 1, these source-points were revisited on multiple transits of portions of the line, with one to seven shots fired at each point. Stacking of these co-located shots clearly improves the signal-to-noise ratio of the data, but we found that profiles consisting of unstacked single shots are of sufficient quality to enable accurate identification and picking of refraction travel times, even at the largest offsets. This result suggests that a very simple shooting strategy may suffice for other experiments of this type.

Velocity modelling

Mantle velocities were determined for line 1 (east of km 300) and line 2 by two-dimensional, ray-trace modelling²⁶ of first-arrival travel times. Sediment thickness and basement topography were constrained using multi-channel seismic data from the IPOD/USGS transect²⁷, which is coincident with the FAIM transect. Crustal thickness and velocity were constrained at the instrument locations using observed crustal phases (Fig. 3). These constraints were used to form the two-dimensional ray-tracing model within which the mantle velocity structure is nearly one-dimensional. The requirement of substantial vertical mantle velocity gradients is indicated by the continuously increasing apparent velocity with offset observed on the Tecate profile, for example, on which the largest-offset first arrivals are observed (Fig. 2).

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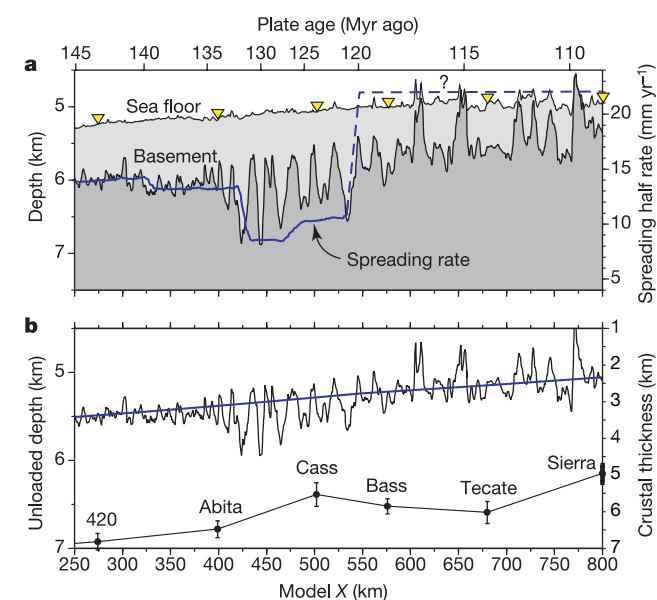


Figure 4 Basement depth, spreading rate and crustal thickness. **a**, FAIM line 1 sea floor and basement depths from multi-beam and multi-channel seismic constraints, and spreading rate⁶. The increase observed at 120 Myr is a jump to the average value for the Cretaceous quiet zone. Note strong correlation between basement morphology and spreading rate. Yellow triangles indicate instrument locations. **b**, Sediment unloaded basement depth, depth predicted from plate cooling (blue line), and crustal thickness estimates for the indicated stations.

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Spatial scaling of microbial eukaryote diversity

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Patterns in the spatial distribution of organisms provide important information about mechanisms that regulate the diversity of life and the complexity of ecosystems^{1,2}. Although microorganisms may comprise much of the Earth's biodiversity^{3,4} and have critical roles in biogeochemistry and ecosystem functioning^{5–7}, little is known about their spatial diversification. Here we

present quantitative estimates of microbial community turnover at local and regional scales using the largest spatially explicit microbial diversity data set available (>10⁶ sample pairs). Turnover rates were small across large geographical distances, of similar magnitude when measured within distinct habitats, and did not increase going from one vegetation type to another. The taxa–area relationship of these terrestrial microbial eukaryotes was relatively flat (slope $z = 0.074$) and consistent with those reported in aquatic habitats^{8,9}. This suggests that despite high local diversity, microorganisms may have only moderate regional diversity. We show how turnover patterns can be used to project taxa–area relationships up to whole continents. Taxa dissimilarities across continents and between them would strengthen these projections. Such data do not yet exist, but would be feasible to collect.

Ecologists studying macroorganisms have long recognized that beta-diversity (how community composition changes across a landscape) is central to understanding the forces responsible for the magnitude and variability of biodiversity. Patterns of beta-diversity can offer valuable clues to the relative influence of dispersal limitation, environmental heterogeneity, and environmental and evolutionary change in shaping the structure of ecological communities^{10–14}. Despite an increasing awareness that spatial patterning of soil microbiota can have important aboveground consequences in regard to plant community structure and ecosystem functioning^{5,6,15,16}, microbial beta-diversity patterns are largely unknown. Inadequate sampling has been a major limitation and scientists are only now beginning to explore emergent patterns and principles that may be common to microbes, plants and animals^{17,18}. Thus, whereas it is widely accepted that the similarity in plant and animal community composition decays with increasing distance between samples^{11,13,19}, patterns of microbial turnover in terrestrial environments remain unstudied. Here, we test whether similarity in microbial eukaryote community composition decays with geographical distance as observed in macroorganisms. We also explore how these biodiversity turnover patterns are influenced by strong habitat-related environmental discontinuities. Finally, we apply spatial scaling theory to these turnover patterns to predict how microbial biodiversity might increase with sampling area from local to continental scales in Australia.

A total of 1,536 soil samples were collected in arid Australia using a spatially explicit nested design. The design resulted in 1,117,880 pairwise sample comparisons, with distances ranging from 1 m to ~100 km represented by multiple replicate sample pairs. Samples were taken from four distinct land systems that varied substantially in geology, topography and native vegetation (see Supplementary Information). We measured the similarity between any two samples using the Sørensen index, defined as the number of taxa in common divided by the average number of taxa in the two samples²⁰. The rate at which Sørensen similarity decays with increasing distance between samples (the distance–decay relationship) can be directly related to the species–area relationship²¹. Other measures of similarity based on presence/absence of data yielded qualitatively similar results to those reported here.

We characterized the beta-diversity of ascomycete fungi by automated ribosomal RNA intergenic spacer analysis (ARISA), a commonly used DNA-based community fingerprinting method^{22–24}. ARISA is a high-resolution, highly reproducible technique for detecting differences between complex fungal communities²². We chose ARISA over DNA sequencing because it allowed assessment of microbial community turnover at an unparalleled sample size and spatial scale. ARISA exploits variability in the length of the intervening transcribed spacer regions of rRNA genes (ITS) to sort samples rapidly into operational taxonomic units (OTUs). Members of different species may share the same ITS fragment size²². Although ARISA assays a different taxonomic resolution than species, it is a consistent measure of community composition

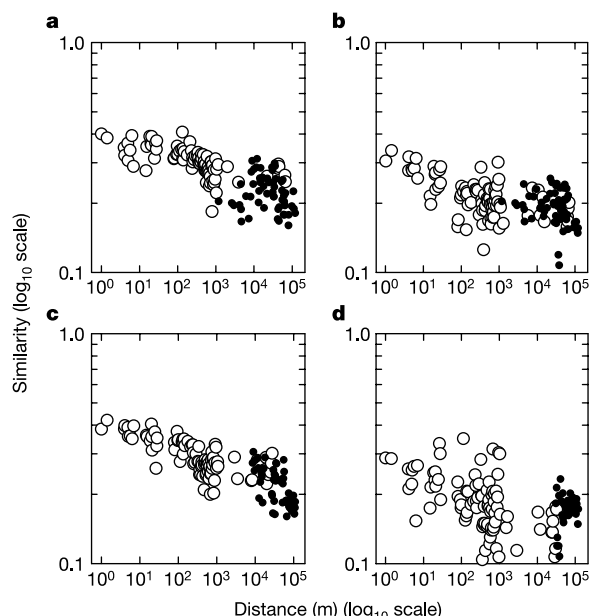


Figure 1 The distance-decay of similarity for microbial fungi OTUs. **a–d**, Shown are the average Sørensen similarity values for within land system data (open circles) and between land system data (filled circles). Averages were taken across similarity values within equidistant logarithmic intervals of 0.01. Data correspond to Pulgamurtie (**a**), Rodges (**b**), Olive Downs (**c**) and Corner (**d**). Summary statistics incorporating the replicate data within each distance class are listed in Table 1.

(that is, members of any one species consistently produce the same fragment size and different-sized fragments always derive from members of one or more different species). Consequently, differences between two OTU assemblages directly reflect changes in species composition.

Within each of the four distinct land systems, community OTU similarity decayed significantly with distance (Fig. 1). These data show that the sampled microbes were not randomly distributed, but rather exhibited spatially predictable, aggregated patterns over scales ranging from 1 m² to 10¹⁰ m². The best fit and the most homoscedastic residuals were found in models that used the log transformation of similarity against the log transformation of distance, implying a power-law rate of distance decay. The average slope estimated from all pairwise replications within land systems was -0.043 (95% confidence intervals (CI): -0.049 to -0.037), and three of the four land systems had slopes that were statistically indistinguishable from one another (Table 1). A prevailing view⁸ expects that microbial eukaryotes such as ascomycetes will prove to be nearly ubiquitous geographically, owing to their large population densities, producing abundant spores that can travel over long distances. The decline in similarity with distance found here demonstrates that there is at least some geographical differentiation.

Geographical distance was a more useful predictor of ascomycete

community turnover than land system type. When the effect of land system was removed, we found a weak but highly significant negative correlation between similarity and geographical distance (Table 1). In contrast, after controlling for geographical distance, we did not consistently find lower similarity between compared to within land system types. For sample pairs with a member in the rocky land systems (Pulgamurtie and Olive Downs), similarity with other samples from the same land system averaged slightly higher than similarity with samples from other land systems, after adjusting for distance; but for sample pairs with a member in the sandy land systems (Corner and Rodges), similarity with other samples from the same land system actually averaged slightly lower than similarity with samples from other land systems, after adjusting for distance. The lack of correlation with land system suggests that ascomycetes respond to their environment at a scale that is poorly described by the geomorphic variables used to classify land systems, being more likely to respond to soil chemistry, water and resource concentrations at much smaller scales.

The slope of the distance-decay curve reflects the rate at which OTU richness increases with sampling area, or the taxa-area relationship. A greater rate of distance-decay between samples implies a faster turnover in OTU composition across a landscape and hence a steeper taxa-area relationship. Recent theory²¹ suggests that if the distance-decay curve in a region is well approximated by a power law, then the taxa-area relationship will also be well approximated by a power law of the form

$$OTU_a = OTU_A \left(\frac{a}{A} \right)^z \quad (1)$$

where OTU_A is the number of OTUs in a region of area A , OTU_a is the number of OTUs in a smaller sampling area a within A , and z is a constant ranging between 0 and 1. The expression relating the distance-decay curve to the power-law OTU-area relationship exponent z takes the form

$$\chi_d = \chi_D \left(\frac{d}{D} \right)^{-2z} \quad (2)$$

where χ_d and χ_D are the expected Sørensen similarities between two samples of equal area separated by distances $d < D$, respectively. Hence the exponent $-2z$ of the power-law distance-decay curve (equation (2)) may be used to estimate the exponent z of the power-law taxa-area relationship (equation (1)) across the spatial scales $a = d^2$ to $A = D^2$. The theory underpinning equations (1)–(2) is equally valid for any biologically consistent taxonomic unit.

The distance-decay curve for the entire microbial data set at Sturt National Park is well characterized by a power-law distance-decay model across the spatial scales d ranging from 1 m to about 10⁵ m. We may therefore estimate the slope z of the OTU-area relationship across these scales. A least squares regression of log-transformed similarity against log-transformed distance through all of the data gives a slope of -0.147 ($n = 907,878$, $r^2 = 0.0051$, $P < 0.001$, 95% CI: -0.152 to -0.143), predicting a power-law exponent $z = 0.074$ across the scales $1 \text{ m}^2 < a < 10^{10} \text{ m}^2$. We can now extrapolate

Table 1 Summary statistics for the fungal OTU distance-decay relationships at Sturt National Park

Land system	Samples	Within land system regression statistics				Partial Mantel correlations		
		n	Slope	r^2	95% CI	N	$r(\text{SD}, L)$	$r(\text{SL}, D)$
Pulgamurtie	381	72,390	-0.047	0.0018	-0.056 to -0.039	442,165	-0.066	0.082
Olive Downs	379	71,631	-0.035	0.0005	-0.047 to -0.024	440,230	-0.097	0.023
Rodges	317	50,086	-0.076	0.0027	-0.089 to -0.064	378,261	-0.091	-0.054
Corner	271	36,585	-0.063	0.0014	-0.080 to -0.046	329,800	-0.087	-0.102

Within land system statistics result from weighted least squares regressions of log-transformed fungal OTU similarity against log-transformed geographical distance. All samples that gave $>1 \mu\text{g}$ of PCR-amplifiable DNA were used in the analysis, and n is the corresponding number of similarity pairs. To account for zero similarity values, logarithmic transformations were of the form $\log(Y + 0.001)^{20}$. All slopes were significantly different from zero ($P < 0.001$); P -values and 95% confidence intervals (CI) are based on 5,000 randomized pairings of OTU similarity and geographical distance²⁸. The partial Mantel statistic $r(\text{SD}, L)$ estimates the correlation between matrices S (OTU similarity) and D (geographical distance) while controlling for the effect of same versus different L (land system). Similarly, $r(\text{SL}, D)$ estimates the correlation between matrices S (OTU similarity) and L (land system) while controlling for the effect of D . Land system matrices L contained ones where pairs of sites were from the same land system and zeros where sites were from different land systems. For any given land system, the number of similarity values N includes the within land system similarities and the similarities between that land system and the other three land systems. All partial regression coefficients were significantly different from zero ($P < 0.001$)²⁸.

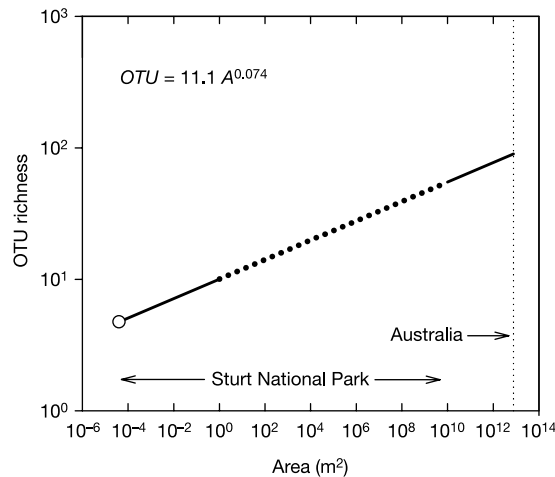


Figure 2 The projected taxa–area curve for microbial fungi. There was an average 4.74 OTUs per $4 \times 10^{-5} \text{ m}^2$ per cm depth in the soil horizon (open circle). The dotted line shows the spatial scales over which the slope $z = 0.074$ is predicted from the observed distance–decay data (equation (2)) and the solid lines illustrate the taxa–area curve that follows from the simple assumption that the slope $z = 0.074$ extends to smaller and larger scales.

microbial biodiversity from the scale of an individual soil sample, where OTU richness is quantifiable, to regional scales using equation (1) (Fig. 2).

It is important to bear in mind that the estimated taxa–area relationship for any group of organisms may depend on the defined OTU. The ARISA-defined OTU used in this study will markedly underestimate the number of Ascomycota species in each soil sample²². The relationship between our estimated slope z and that derived using different taxonomic criteria, such as whole-genome relatedness or morphological similarity, is less clear. Our predicted taxa–area slope is remarkably consistent with those reported for microbial eukaryotic species in the Arctic benthos⁹ (ciliates $z = 0.077$, diatoms $z = 0.066$) and in freshwater habitats²⁵ (ciliates $z = 0.043$). Thus, studies using different taxonomic criteria indicate that despite high local diversity, microbial eukaryotes may have only moderate spatial turnover and hence moderate regional diversity. It remains possible that z increases between regional and continental (or global) scales as new and distinct habitats are included or if dispersal barriers are crossed. Notably, no reliable data currently exist that would allow global-scale extrapolations of microbial diversity, and further large-scale quantification of community turnover is essential for improving microbial biodiversity estimates.

Many ecologists have treated the entire microbial community as a “black box” with no spatial structure¹⁵. Our data illustrate that like macroorganisms, microbial eukaryotes are not randomly distributed, but rather exhibit spatially predictable, aggregated patterns from local to regional scales. We have shown that by sampling localities spatially, in such a way that the decline in similarity with distance can be measured, the slope z of the taxa–area relationship can be estimated, leading on to estimates of the total diversity over wide areas. The relationship between biodiversity and area is central to ecology. Despite the ecological importance and ubiquity of microorganisms, little is known regarding microbial taxa–area relationships. Our findings offer exciting potential for a more synthesized view of micro- and macroorganism biodiversity, and ultimately a means to estimate global biodiversity much more accurately. □

Methods

Sampling methods and DNA extraction

The study area was within Sturt National Park, New South Wales, Australia. ‘Land system’ mapping undertaken by the New South Wales Soil Conservation Service made this region

ideal for studying the influence of habitat type on microbial beta-diversity. Within the park, 23 land systems with distinct patterns of topography, soil and vegetation had been previously mapped. Four of these land systems, representing 42% of the area of the park, were included in the study. These were Olive Downs (stone-covered rolling downs), Pulgamurtie (stony uplands), Corner (sand dunes) and Rodges (sand plains). The soils, geology and vegetation in each land system, and the basis for choosing each land system type, are described in more detail by ref. 26.

Six locations within each of the four land systems were selected to provide a total of 24 study sites. Each study site comprised a $750 \times 750 \text{ m}$ plot with 64 sampling points, yielding a total of 1,536 sampling points and $1,536 \times 1,535/2 = 1,178,880$ sample pairs (see Supplementary Information). In September/October 1997, at each of the 1,536 sampling points a soil sample of approximately 10 g was taken at a depth of 8 cm below the ground surface and frozen on site.

Ascomycetes were sampled by collection of their DNA from soil. For DNA extraction, the total soil sample (10 g) was homogenized using a sterile mortar and pestle before a 400-mg subsample was taken for DNA extraction using a variant of the FastPrep bead beating method, as described previously²⁷. The DNA yield was typically from 2.0 to 2.5 μg in a volume of 160 μl (see Supplementary Information).

ARISA

The primers SSU1758 (5′-GTCATTAGAGGAAGTAAAGTCG-3′, positions 1735–1758 of the SSU rRNA gene, *Saccharomyces cerevisiae* numbering) and 58S8 (5′-CAGAACCAAGATCCGTTGTG-3′, positions 30–8 of the 5.8S rRNA gene, *S. cerevisiae* numbering) were used for amplification of the ITS1 region. This primer pair selectively targets ascomycete fungi in PCR (see Supplementary Information). Amplifications were performed in 50- μl volumes using 0.5-ml tubes in a Hybaid Omne thermal cycler with the following thermal cycle: 94 °C for 3 min (1 cycle); 94 °C for 30 s, 62 °C for 15 s, 72 °C for 60 s (35 cycles); 72 °C for 5 min (1 cycle). The banding profile from individual soil samples was reproducible when either multiple DNA extractions were performed on subsamples from the same soil sample, or multiple PCRs were performed on the same DNA sample (see Supplementary Information). The size range of amplicons was ~170 to ~800 base pairs (bp), with approximately 80% being in the size range of 200–350 bp. For electrophoretic separation, a Corbett GS 2000 DNA fragment analyser (Corbett Research) was used with high-resolution, ultrathin 5% polyacrylamide gels and DNA fragments detected by laser. Data were analysed using the RFLPscan package (Scanalytics). Size of DNA fragments was determined using the desmole method in reference to a 50-bp ladder (Pharmacia) loaded as a standard every eighth lane.

Fragments differing by 1 bp were readily resolved in one gel, and the relative error of their measured length was 2%. For comparisons between gels, we accounted for this by sorting the electropherogram data into bins of size Y_{bin} , where $X < Y_1 < \text{int}(X + 0.02X)$, $\text{int}(X + 0.02X) < Y_2 < \text{int}(X + 0.04X)$, and so on. Here, X denotes fragment length and int denotes integer value. The observed DNA fragments were resolved from 130 bp to 568 bp, yielding a total of 69 OTU bins. The bin presence/absence data were then used for subsequent calculations of the Sørensen index, beta-diversity and taxa–area relationships.

Partial Mantel tests

Several extensions to the basic Mantel randomization test are sensitive to spatial autocorrelation²⁸. Partial Mantel statistics were therefore estimated using the ‘matrix permutation’ method, which has been shown to perform the most reliably in the presence of spatial autocorrelation, being unlikely to reject the null hypothesis falsely when a conservative critical value is used^{20,29,30}.

Taxa–area relationship estimates

Numbers of OTUs per volume of soil were translated into numbers of OTUs per m^2 in a 1-cm-depth layer in the soil horizon by assuming 1 g cm^{-3} soil bulk density. Although each 400-mg sample was randomly drawn from the surrounding 10 g of soil, equation (2) holds assuming that equal numbers of individuals were drawn per sample.

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A taxa–area relationship for bacteria

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A positive power-law relationship between the number of species in an area and the size of that area has been observed repeatedly in plant and animal communities¹. This species–area relationship, thought to be one of the few laws in ecology², is fundamental to our understanding of the distribution of global biodiversity. However, such a relationship has not been reported for bacteria,

and little is known regarding the spatial distribution of bacteria, relative to what is known of plants and animals³. Here we describe a taxa–area relationship for bacteria over a scale of centimetres to hundreds of metres in salt marsh sediments. We found that bacterial communities located close together were more similar in composition than communities located farther apart, and we used the decay of community similarity with distance to show that bacteria can exhibit a taxa–area relationship. This relationship was driven primarily by environmental heterogeneity rather than geographic distance or plant composition.

In the 1920s, the empirical relationship between the number of species and area was generalized^{4,5} as a power-law, $S = cA^z$, where S is the number of species, A is the area sampled and c is the intercept in log–log space. The species–area exponent, z , is a measure of the rate of change of the slope with increasing area, that is, the rate of turnover of species across space. Variation in the values for c , and especially for z , is of interest because it may indicate that different processes underlie the species–area relationship at different spatial scales^{6,7}. Although not as well studied as species–area relationships, other taxa–area relationships (for example, genera–area and family–area) have been identified for plants and animals; such relationships conform to the same power-law as species–area relationships, although they differ in their values of c and z ^{8,9}.

Bacteria are among the most abundant and diverse groups of organisms on earth¹⁰ and mediate important ecosystem processes, including trace gas emissions, decomposition and nitrogen cycling. Whereas taxa–area relationships have been observed repeatedly for numerous plant and animal taxa regardless of ecosystem type¹, they have not been explicitly examined for bacteria. Unique aspects of bacterial biology may prevent bacteria from exhibiting taxa–area relationships. For example, if most bacteria are not dispersal limited (for example, owing to small size and environmental hardness)¹¹ and if they exhibit a high degree of ecological redundancy (for example, if bacteria are flexible in habitat requirements and physiological abilities, or if they can easily obtain traits through horizontal gene transfer that are necessary for survival in a given habitat), then one would not expect to observe a taxa–area relationship³.

Here we investigated whether bacteria exhibit a taxa–area relationship in a New England salt marsh. We conducted our work in a salt marsh because the spatial ecology of salt marshes is especially well understood¹². There is an extensive literature regarding the main physical gradients in salt marshes, the spatial distribution of plant species and the ecological processes that underlie this distribution. This information provides an ideal reference point from which to investigate the spatial distribution of bacteria. We sampled 1-cm-diameter sediment cores in a nested manner over a scale of centimetres to hundreds of metres. With the possible exception of the most extreme and depauperate environments¹³, the diversity of bacterial communities is too high to be exhaustively sampled. Therefore we used a previously refined distance decay approach¹⁴, which uses data on the spatial turnover of taxa, to determine the taxa–area exponent, z . This approach uses comparisons of community composition rather than richness estimations to describe taxa–area relationships. For comparison, we also estimated the relationship between the number of plant species and area in this ecosystem, using the same distance decay approach.

Because a large proportion of microbes cannot be cultured with current laboratory techniques¹⁵, bacterial taxa are often identified from the sequences of indicator genes extracted from environmental samples¹⁶. We determined the bacterial community composition of our salt marsh samples by amplifying via the polymerase chain reaction (PCR), cloning and sequencing a region of 16S ribosomal DNA (rDNA), the most commonly used indicator gene for bacterial biodiversity. Because the bacterial diversity of salt marshes is often very high, we used PCR primers targeting a subset of the bacterial

biota (the β -proteobacteria and relatives) to constrain the potential community we sampled. Proteobacteria are commonly found in many different environments, including salt marshes, and are often numerically dominant¹⁷. Because there is no single best definition of 'species' using this sequencing approach¹⁸, taxa are usually defined as operational taxonomic units (OTUs) based on sequence similarity groupings. We used the three most commonly used groupings—95%, 97% and 99% sequence similarity—to define OTUs in our study. Using multiple OTU definitions is analogous to comparing different taxonomic resolutions (for example, comparing genus, species and sub-species)¹⁶.

We sequenced a total of 945 partial 16S rDNA sequences. These sequences grouped into 88 OTUs, using a taxon resolution of 97% sequence similarity. Approximately 34% of the OTUs were singletons ($n = 30$), and 15 OTUs were represented by more than ten sequences. Five-hundred and twenty-three sequences were members of the β -proteobacteria; the remainder were members of the γ -proteobacteria and the δ -proteobacteria.

Bootstrapping of linear regressions between log-transformed values of bacterial similarity and geographic distance revealed significant distance decay curves for all taxonomic resolutions; that is, samples that were located closer in space were significantly more similar in bacterial composition than samples that were located farther apart (Table 1; see Supplementary Methods 2). We then computed the taxa-area z -value for bacteria from the slope of these distance decay curves (Table 1; Fig. 1).

We also observed a significant species-area relationship for plants in this same marsh (Fig. 1a). The plant z -value was significantly larger than those observed for bacteria but was similar to values estimated for other wetland plant communities¹⁹. To determine whether taxonomic focus (the particular group of taxa analysed) also affects the taxa-area relationship among different bacterial taxa, we repeated the analyses above for a subgroup of the sequences, restricting our analyses to the β -proteobacteria

(Table 1). The z -value for the 99% sequence similarity grouping of the β -proteobacteria was significantly lower than that of the entire group of sequences at the 99% resolution (Fig. 1b). This suggests that the turnover in space of β -proteobacteria was lower than that of the other bacteria we sampled.

The z -value also varied by taxonomic resolution, increasing with increased taxonomic resolution for bacteria. The estimated z -value for all sequences at the 99% resolution was significantly larger than those estimated at the 97% and 95% resolutions. Similarly, the z -value at the 99% resolution for β -proteobacteria had a significantly positive z -value, whereas the z -values at the 97% and 95% resolutions were not significantly different from zero (Table 1; Fig. 1b). A similar increase in z -values with increasing taxonomic resolution has been observed for plants⁸ and animals⁹ (that is, from family to genera to species). We expect that the z -value will continue to change with taxonomic resolution at both higher and lower resolutions. We did not present the 100% sequence similarity OTUs because of the possibility that even minor PCR and/or sequencing errors could result in artefactual OTUs at this resolution. The taxonomic resolutions we used probably include a greater diversity of ecological types than contained within an animal or plant species. However, the presence of a taxa-area relationship for bacteria at this relatively coarse resolution suggests that a taxa-area relationship would probably also be present at finer resolutions, resolutions that may more closely correspond to the ecological breadth of animal and plant species.

Environmental heterogeneity often increases with increased area. The increase in heterogeneity with area together with the specificity of taxa for different habitats is the most common explanation of taxa-area patterns¹, especially at scales where dispersal is not limiting to the distribution of taxa. Environmental heterogeneity may also underlie the bacterial taxa-area relationship observed here. When we removed the effect of geographic distance, partial Mantel tests showed that sites that were similar in environmental charac-

Table 1 Taxa-area z -values

OTU resolution	z -value	Regression coefficient	t	P
All sequences				
99%	0.040	-0.081	-6.53	<0.001*
97%	0.020	-0.039	-4.92	<0.001*
95%	0.019	-0.037	-5.44	<0.001*
β -proteobacteria				
99%	0.019	-0.038	-2.40	0.013*
97%	0.048	-0.096	-1.14	0.396
95%	0.008	-0.015	-1.57	0.177

The z -values shown were determined using the distance decay approach. Asterisks denote regressions that were significant at the $P < 0.05$ level. The z -value was determined as $-(\text{regression coefficient})/2$. Percentages refer to the 16S rDNA sequence similarities assumed when defining taxonomic units.

Table 2 The influence of geographic distance and habitat heterogeneity on bacterial community composition

Effect of: Controlling for:	Env. simil. Geog. dist.		Geog. dist. Env. simil.		Plant simil. Env. simil.	
	r	P	r	P	r	P
All sequences						
99%	-0.36	0.0002*	-0.15	0.11	-0.069	0.17
97%	-0.37	0.0001*	-0.0089	0.46	-0.10	0.080
95%	-0.36	0.0002*	-0.056	0.27	-0.085	0.13
β -proteobacteria						
99%	-0.32	0.0004*	0.012	0.47	-0.043	0.27
97%	-0.29	0.0004*	0.11	0.11	-0.022	0.35
95%	-0.26	0.0009*	0.082	0.17	0.0014	0.51

Partial Mantel tests showed that environmental heterogeneity may underlie the bacterial taxa-area relationship. Sites that were similar in environmental characteristics (env. simil.) were similar in bacterial composition when we removed the effect of geographic distance (geog. dist.) and plant community composition (plant simil.). r represents the Mantel statistic, and asterisks denote significance at the $P < 0.05$ level (9,999 permutations).

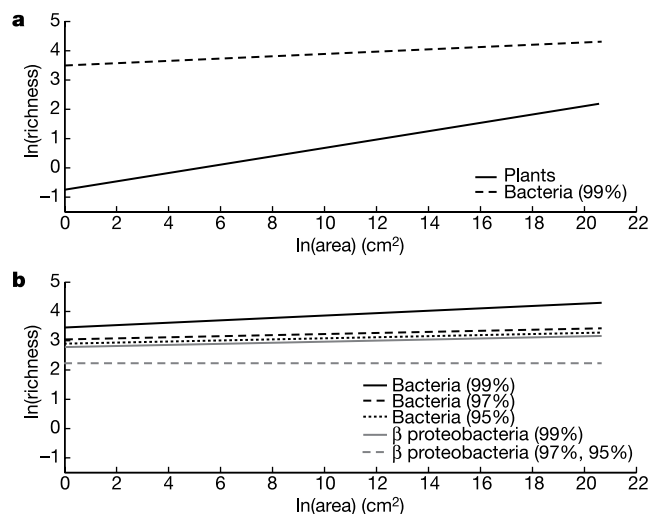


Figure 1 The taxa-area relationship for salt marsh organisms varied with taxonomic focus (a) and taxonomic resolution (b). a, Plants and bacteria both exhibited significant taxa-area relationships, but the z -value for plants ($z = 0.103$) was significantly greater than the z -value for bacteria ($z = 0.040$; coeff. = 0.0198, $t = 159.51$, $P = 0.001$). b, Both taxonomic resolution and taxonomic focus influenced the taxa-area relationship for salt marsh bacteria. Lower resolution OTUs (that is, those defined using the criteria of 97% or 95% sequence similarity) had a lower slope ($z = 0.020$ and $z = 0.019$, respectively; coeff. = -0.041, $t = -3.32$, $P = 0.0008$) than the higher resolution OTUs (99% sequence similarity). The β -proteobacteria only exhibited a significant taxa-area relationship for the 99% resolution (grey solid line, $z = 0.019$).

teristics were also similar in bacterial composition (Table 2). In contrast, there was no effect of geographic distance on community similarity when we removed the effect of environmental similarity (Table 2). Although there was a significant relationship between environmental similarity and plant community composition ($r = 0.36$, $P < 0.001$), there was no relationship between plant similarity and bacterial similarity independent of environmental similarity (Table 2).

The z -values for bacteria reported here are among the lowest z -values reported for any organisms (Fig. 2), suggesting that turnover of bacterial taxa at these spatial and taxonomic scales may be lower than that of most other organisms. Bacterial z -values may be low, in part, because it is unlikely that bacteria are dispersal limited within a salt marsh. Bacteria can probably disperse easily by air and tidal waters at this scale, and indeed we found no evidence for an effect of geographic distance on bacterial community composition when controlling for environmental heterogeneity and plant community composition. However, it is unlikely that the plants, which have a z -value at least two times higher than the bacteria, are dispersal limited at these spatial scales either, suggesting that dispersal alone cannot be responsible for the low bacterial z -values. We can think of at least three other potential explanations. First, the levels of taxonomic resolution we examined for bacteria probably reflect a broader ecological breadth than the plant species units, and thus the lower z -values for bacteria may be a reflection of taxonomic resolution. In other words, if we could define bacterial OTUs in terms that were equivalent to the ecological breadth of a typical plant species, then perhaps the bacterial species–area curve would have a z -value similar to that observed for many macro-organisms. Second, although environmental factors were related to community composition, the bacteria we sampled could have low habitat specificity (relative to the plants), reducing the spatial turnover of taxa relative to the plants in this system. Third, horizontal transfer of ecologically relevant genes could uncouple the relationship between phylotypes (phylogenetically distinct groups) and ecotypes (ecologically distinct groups), resulting in a lower z -value³.

We saw no evidence that the bacterial z -value was scale-dependent (that is, the slope of the distance decay curve did not vary with distance). However, it is possible that the z -value may

increase at larger spatial scales, as has been observed for plants⁶, or even at smaller scales, where microscale environmental heterogeneity can be very high³. Indeed, recent reports demonstrate that dispersal limitation is possible at large spatial scales for some microbes²⁰ (although not for all²¹), and this would tend to increase the z -value.

For over two-hundred years, ecologists have repeatedly documented that there is a relationship between the number of eukaryotic species found in a given area and the size of the area. Our study demonstrates that both prokaryotes and eukaryotes can exhibit taxa–area relationships, although the quantitative form of the relationship may differ. The taxa–area relationship seems to be a universal law. □

Methods

Sampling

We collected samples from a salt marsh on Prudence Island, Rhode Island in July 2002. Twenty-six sediment samples for bacterial community composition were collected from a nested grid that ranged from 300 × 300 m to 0.03 × 0.03 m (see Supplementary Methods 1).

We sampled plant community composition in two ways. First, we sampled along three transects at the 300-m and 30-m scales, with transects spaced at 100-m and 10-m intervals, respectively. We recorded the presence and per cent cover of each species in 1 × 1 m quadrants every 20 and 10 m, respectively, and used these data to determine the plant species–area relationship. Second, we recorded similar data in a 10 × 10 cm quadrant at each bacterial sampling point and used these data to examine the influence of plant composition on bacterial community composition.

We collected porewater samples adjacent to each sediment core to measure salinity, pH and nutrient concentrations (phosphate, ammonium and sulphate). Water was filtered with a 40-micron filter immediately after collection. Sulphate, ammonia and phosphate were measured in the field using Hach portable colorimeters (<http://www.hach.com>). Salinity was measured with a refractometer and pH with a calibrated probe.

DNA analyses

DNA was extracted from the top 0.5 g of each sediment core using a combination of phenol/chloroform extraction and a MoBio Ultraclean Soil DNA Kit (MoBio Laboratories). We chose primers (β AMOf and β AMOr) that amplify 16S rDNA from a subset of the domain Bacteria, as it is not tractable to sufficiently sample bacteria in our samples using domain level primers²². We reduced PCR biases by limiting the PCR cycles to twenty-five and included BSA in the reaction mix²³. Each cycle consisted of 30 s at 94 °C, 30 s at 57 °C and 90 s at 72 °C. Gel-extracted and purified amplicons were cloned using the TOPO-TA cloning kit for sequencing (Invitrogen). We used an ABI 377 automated DNA sequencer to determine the sequence of the 5' terminal 600 nucleotides of 945 of the cloned rDNA amplicons.

Phylogenetic analysis

We used the RDP database²⁴ and ARB software²⁵ to align the rDNA sequences from our 26 clone libraries. Ambiguously and incorrectly aligned positions were aligned manually on the basis of conserved primary and secondary structure. We identified and excluded potential chimeras using the Chimera_Check program of the RDP²⁴ and using ARB to compare trees generated from the 5'-end versus the 3'-end sequences separated at the break point suggested by Chimera_Check. Similarity matrices were generated using 510 unambiguously aligned positions. We grouped sequences into OTUs on the basis of rDNA sequence similarity, using DOTUR²⁶. We used the three most commonly used groupings—95%, 97% and 99% sequence similarity—to define OTUs in our study and to examine how the taxa–area relationship varies with taxonomic resolution.

Community similarity

First, we calculated turnover using the Sorensen index, for use in estimating the z -values¹⁴. Second, we calculated the Bray–Curtis similarity coefficient (S_{BC}) for each pair of samples, for use in the Mantel tests²⁷. We calculated S_{BC} on square-root-transformed data to decrease the influence of highly dominant sequences, because the most dominant sequences in a clone library may not be the most active or dominant types in the actual community, owing to primer bias²⁸.

To control for unequal sampling (numbers of sequences) between sediment cores, we used a form of community rarefaction when calculating the pairwise community similarity indices. We randomly sampled the lowest number of sequences found at any point from each core and calculated the similarity values between all cores. We then repeated this randomization 1,000 times to get a rarefied community similarity matrix.

Taxa–area relationships

We used linear regression to examine the relationship between geographic distance between samples and similarity in bacterial composition. Because our data consisted of pairwise comparisons and thus were not independent, we used bootstrapping (10,000 replications) to test if the slope of the regression was significantly different from zero (see Supplementary Methods 2). We estimated the power-law exponent z with a distance decay approach¹⁴, using the equation $\log(S_s) = \text{constant} - 2\log(D)$, where S_s is pairwise similarity in community composition and D is distance between two samples. Because

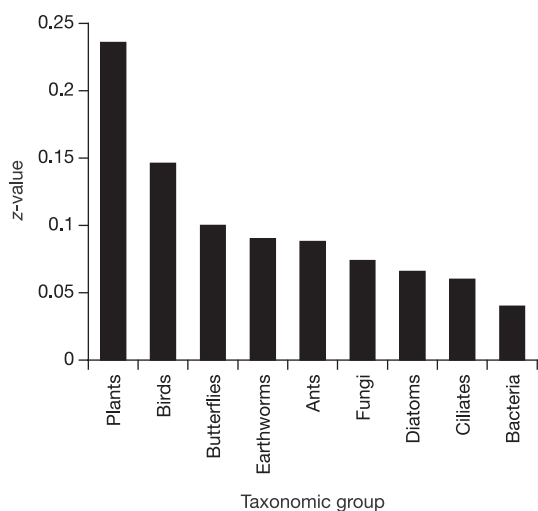


Figure 2 A comparison of z -values for both microbial and macrobial taxa in different ecosystems. The bacterial z -values observed in this salt marsh system represent some of the lowest values observed so far. Representative z -values were selected from a review of taxa–area relationships (see Supplementary Methods 3)

some similarity values were equal to zero (that is, no OTUs in common), we coded the similarity data by adding 0.01 before log transforming each value²⁹. The approach outlined in ref. 14 allowed us to use relative comparisons of bacterial community composition rather than richness to examine the taxa–area relationship; richness is very difficult to estimate accurately in hyperdiverse communities such as bacterial communities. There is no reason to believe that undersampling and/or PCR biases will co-vary with intersample distance or will result in preferential sampling of those taxa most likely to be shared among samples located close together in space; thus, these factors, although likely to be present, are unlikely to influence the z -values we observed. In addition, we can think of no model in which PCR biases and/or undersampling could generate a taxa–area relationship that was completely artefactual.

We compared the z -values for different taxon definitions by testing if the slopes of the regressions differed (see Supplementary Methods 2). We used the same distance decay approach to determine the z -value for plants, using data from the transect quadrants.

Influence of habitat heterogeneity

We used partial Mantel tests (9,999 permutations) to examine the influence of abiotic factors and aboveground plant composition on bacterial community composition, while holding geographic distance constant and vice versa.

We constructed a distance matrix for plant community composition from per cent cover estimates for the four dominant species (*Spartina alterniflora*, *Spartina patens*, *Salicornia virginica* and *Limnium nashii*). We constructed a matrix of environmental distance from the abiotic factors identified as most important to community composition (phosphate and ammonia concentrations), using BIO-ENV²⁸. The BIO-ENV procedure selects a subset of available abiotic variables to maximize rank correlation between community similarity and abiotic dissimilarity matrices. We then used these matrices to test for additional distance and plant effects.

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Species-typical songs in white-crowned sparrows tutored with only phrase pairs

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Modern theories of learned vocal behaviours, such as human speech and singing in songbirds¹, posit that acoustic communication signals are reproduced from memory, using auditory feedback². The nature of these memories, however, is unclear. Here we propose and test a model for how complex song structure can emerge from sparse sequence information acquired during tutoring. In this conceptual model, a population of combination-sensitive (phrase-pair) detectors is shaped by early exposure to song and serves as the minimal representation of the template necessary for generating complete song. As predicted by the model, birds that were tutored with only pairs of normally adjacent song phrases were able to assemble full songs in which phrases were placed in the correct order; birds that were tutored with reverse-ordered phrase pairs sang songs with reversed phrase order. Birds that were tutored with all song phrases, but presented singly, failed to produce normal, full songs. These findings provide the first evidence for a minimal requirement of sequence information in the acoustic model that can give rise to correct song structure.

Songbirds must hear conspecific song during a 'sensitive period' early in life to later generate a normal song; birds fail to produce normal song if raised from the egg or the nestling stage, in acoustic isolation^{3,4}. From this early acoustic experience, birds form a memory (acquired template) of the song^{5,6}. Later, during the sensorimotor phase of song development, birds use auditory feedback to compare their vocalizations with the memorized representation of the tutor song². The neural representation of this acquired template is largely unknown.

A central question with regard to the nature of the acquired template is whether a representation of the full song (Fig. 1a) is required for assembling normal song. Here we propose and test a model of how correct song structure could emerge even if the full song was never experienced. Theoretically, correct phrase order could be deduced if only information about the temporal order of adjacent phrases is represented separately in the tutor model (as phrase pairs, Fig. 1b, c), but not when birds are tutored with phrase types presented in temporal isolation. In support of the latter, Soha and Marler⁷ found that white-crowned sparrows (*Zonotrichia leucophrys*) tutored with multiple models, each consisting of repe-

titions of only a single phrase type, failed to produce normal, full songs. Birds tutored with phrase pairs, however, might be able to assemble full song; auditory experience could shape neural selectivity for phrase pairs present in tutor models. These sensory units, representing the acquired template, could then be activated by auditory feedback from self-generated vocalizations and provide reinforcement for 'correct' motor patterns (for additional details of this model, see Supplementary Information).

According to this model, therefore, birds should be able to develop full, dialect-specific song if tutored with pairs of phrases that are normally adjacent, but not when tutored with phrases presented in isolation. We tested these predictions by tutoring (see Methods) white-crowned sparrows, 10–14 days old, with either singly presented phrases or one of two phrase-pair regimens (Fig. 1b, c). The white-crowned sparrow is an excellent system for evaluating this model because its song consists of four or five acoustically distinct segments (phrases, labelled ABCDE, Fig. 1a), a long tradition of research on song learning exists for this species and the phrase sequence varies between subspecies and dialects^{8,9}—with the exception of the whistle, which is always in the first position. Birds in the first phrase-pair tutored group were exposed to the natural phrase order (forward-order) within each phrase pair; that is, the order found in the natural song. For the other group, the phrase order was reversed (reverse-order). Phrase pairs were presented in an order such that the full forward or reversed sequence of phrases was not present across pairs. We predicted that birds in the forward-order group would develop full songs that had the natural phrase order, whereas those in the reverse-order group would construct songs with phrases in the opposite order. Birds tutored with temporally isolated phrases of all types should not, however, assemble normal songs.

Nine birds that were tutored with singly presented phrases produced crystallized songs. Across individuals, songs consisted of two to four phrase types (median, 3; Fig. 2), were of approximately normal length (median, 2.0 s; range, 1.6–2.2) and had significant forward phrase order ($P < 0.02$, binomial test). No birds, however, assembled songs of four to five phrase types placed in the correct order. The forward structure of these songs stemmed principally

from the innate predisposition^{4,7} of white-crowned sparrows to begin songs with one or more whistles (eight of nine cases), and a tendency (six of nine cases) in this study to follow an initial whistle with a B-type phrase (note complex) ($P < 0.01$, binomial test). The inclination to place the B in the second position, however, was weak; even as late as 3 weeks before crystallization, seven of the birds still repeatedly sang some songs (variants, Fig. 2) in which B did not follow the initial whistle. These results generally support the idea that birds have some inclination to assemble songs that have several different phrase types, that is, use a 'phrase diversity strategy'. Birds did not, however, include more than three representatives of the phrase types in the tutor models; most notably, C-type phrases were absent. These findings are in general agreement with those of Soha and Marler⁷, except that the crystallized songs of birds in their study consisted, on average, of two phrase types. These results support the notion that birds have an innate predisposition to produce songs of normal length that begin with whistles, and a tendency to follow the whistle(s) with a note complex, but lack an innate capacity to assemble component phrases into complete, normal songs.

According to the model, however, birds should be able to develop full, dialect-specific song if only tutored with pairs of phrases that are normally adjacent. We recorded song from five birds in the forward-order tutoring regimen. Four birds developed songs with a natural sequence of at least four phrases (ABCD) (Fig. 3). Under the experimental conditions used in this study, it is not uncommon that first-year birds do not produce the last syllable (E, in this case) in their crystallized song¹⁰. The fifth bird (bird number 45) developed a song, in which only two of the tutor syllables (AB) were combined with an improvised syllable; because only two phrases from the tutor models were copied, the song of this bird was inadequate for testing the present hypothesis. Nevertheless, as a whole, these data show that the phrase pair information in the tutor models was sufficient for birds to assemble songs with correct phrase order ($P < .001$, binomial test). Also, as a group, the songs of these birds contained significantly more forward structure than those of birds tutored with single phrases ($U = 38$, $P < .025$, Mann–Whitney U -test).

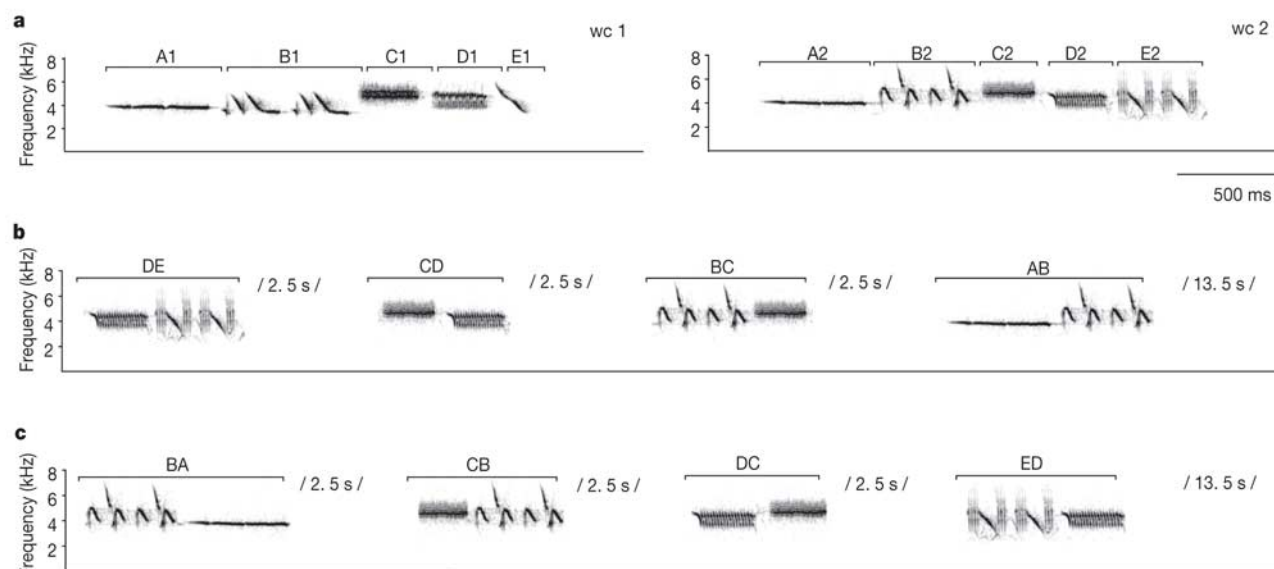


Figure 1 Sound spectrograms of songs of two white-crowned sparrows and tutor models. **a–c**, Songs consist of four to five segments, referred to as 'phrases', denoted as A, B, C, D and E. Phrases generally are composed of repeated notes or syllables. Tutoring regimens consisted of single phrases (not shown) or pairs of phrases from the songs shown in **a**.

Paired phrases were either in the normal (**b**) (for example, AB) or reversed (**c**) order; phrases in these pairs were always from the same song. Only the phrase pairs taken from the song of bird 2 are shown. wc 1 and wc 2, indicate the songs of birds 1 and 2, respectively.

Thus, white-crowned sparrows can assemble apparently normal songs despite being tutored with only pairs of phrases; exposure to the full song is not necessary. This process was undoubtedly aided, however, by the innate predisposition of these birds to begin their

songs with whistles⁷, and the tendency to follow the whistle with note complexes (Fig. 2). The second group, tutored with reverse-order pairs, further tested therefore the importance of phrase order in the tutor pairs as a determinant of song phrase-sequence development.

We recorded song from five males tutored with reverse-order phrase pairs. By the late plastic song stage, four of these birds (67, 72, 74 and 84) assembled songs with the full sequence of phrases, in reversed order (EDCBA, Fig. 4a). Of the 101 songs recorded and analysed that consisted of all five phrase types, 78 were EDCBA sequences ($\chi^2 = 7167$, $P < .001$); no ABCD or ABCDE songs were recorded.

The crystallized songs of these birds generally also consisted of phrases in reversed order, but were less complete than those shown in Fig. 4a. Bird 67 crystallized a DCBA song (Fig. 4b). Birds 72, 75 and 84 also failed to retain their EDCBA songs, but crystallized songs that lacked C-type phrases. The fifth bird (not shown) produced an improvised introductory syllable, followed by either ABA or ADB. Collectively, the songs of birds in this tutoring group showed significant reversed phrase order ($P < 0.001$, binomial test). That four of the five birds crystallized songs that ended with BA ($P < 0.001$, binomial test), is remarkable in light of their predisposition to position whistles at the beginning of the song⁷ (Fig. 2). Apparently the early experience with reverse-ordered

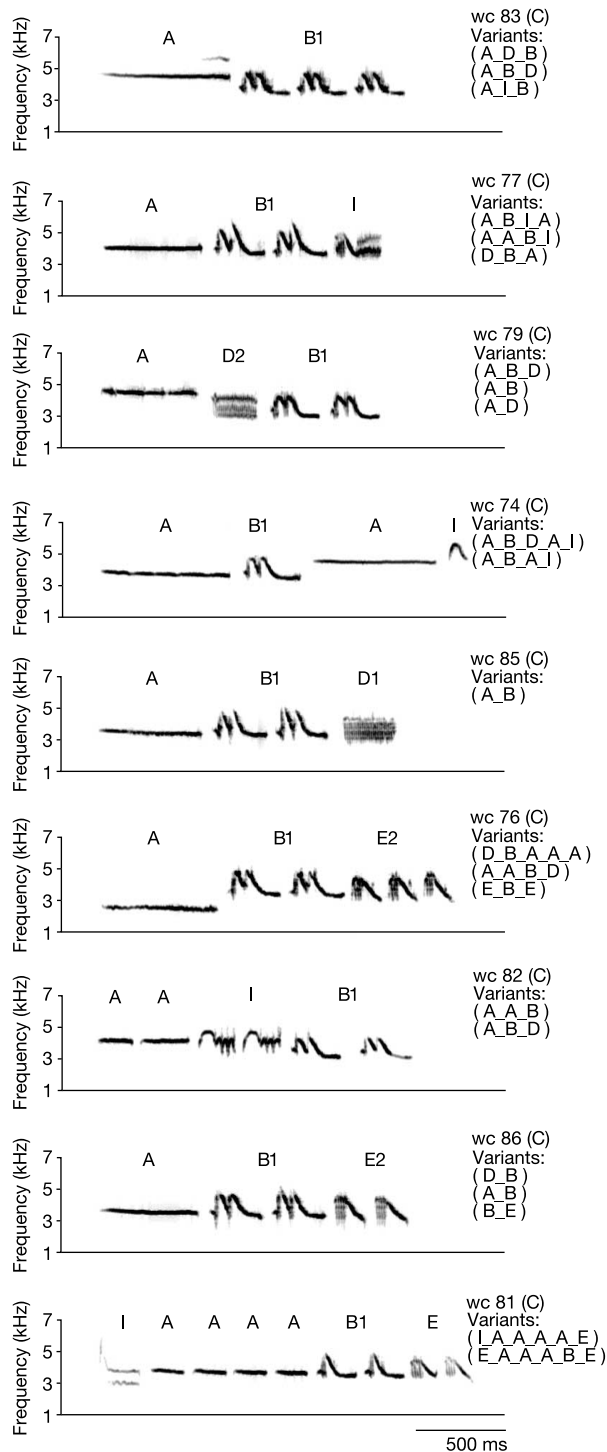


Figure 2 Sound spectrograms of representative crystallized songs. Birds were tutored with the phrase types shown in Fig. 1, but presented singly and in the order E, D, C, B, A. The songs (variants) that each bird sang before crystallization (C) are denoted by the letter sequences to the right of each spectrogram. Phrases in spectrograms are labelled with respect to the tutor phrases that they represent; improvised phrases (I) either did not appear to match a tutor phrase, or were amalgamations of two tutor phrase types.

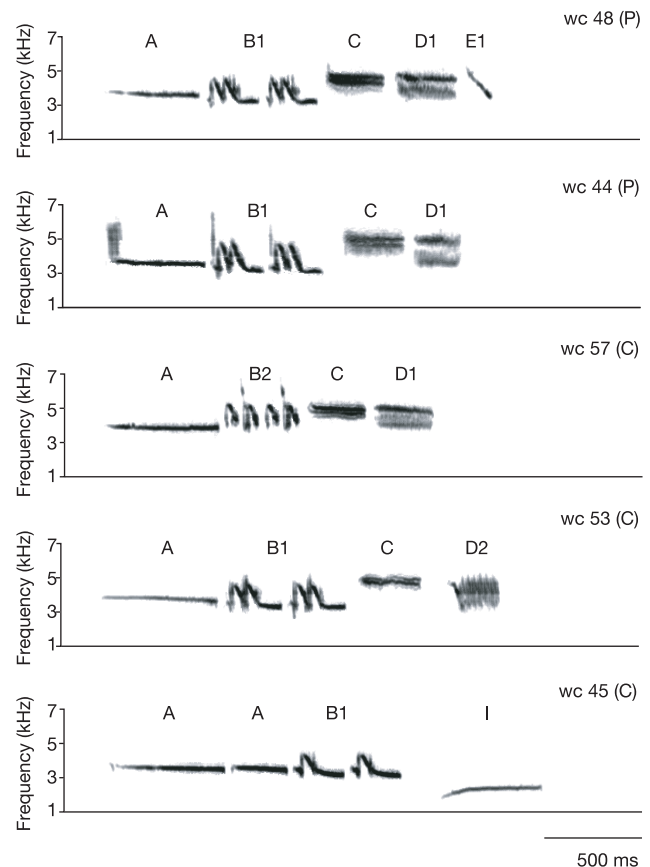


Figure 3 Sound spectrograms of representative songs of birds that were tutored with the phrase pairs DE, CD, BC and AB, presented in that order (see Fig. 1b). Late in the singing season, birds sang predominantly a single 'crystallized' (C) song. The song of bird 48, recorded during the late plastic-song stage (P), is shown to demonstrate that this bird was able to assemble a complete wild-type phrase sequence, despite ultimately crystallizing an ABCD song. Bird 44 died before crystallization, hence his late plastic-stage song is shown.

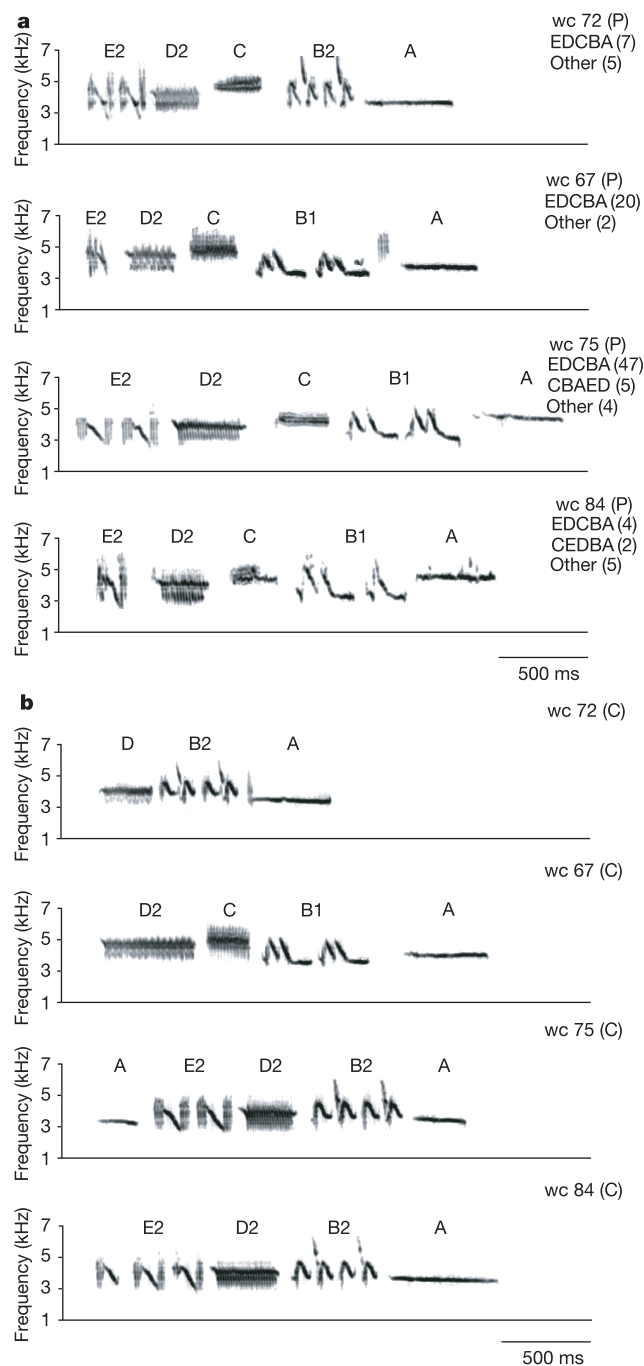


Figure 4 Sound spectrograms of representative songs of birds that were tutored with the phrase pairs BA, CB, DC, ED, presented in that order. The order of phrases in each pair, therefore, was reversed from that seen in wild-type songs. As before, (C) and (P) refer to crystallized and plastic song. **a**, Full reversed phrase-order songs produced by four of the birds during plastic song. Numbers in parentheses indicate the number of songs consisting of all five phrase types produced by each bird; single cases of particular types were grouped as 'others'. For bird number 67, 21 of the songs were recorded during the plastic stage of his second year. **b**, Crystallized songs of the same four birds.

phrases largely overrides the innate tendency to start songs with whistles.

How did birds arrive at their respective songs? Our model posits that some time early in song development, while still perfecting syllable and phrase structure, birds begin to combine phrases; first, phrases are assembled primarily into pairs, then into longer

sequences. The model also posits that, because only phrase pairs that match those in the tutor models can be reinforced maximally, over time, birds retain and refine these sequences, while eliminating other phrase pair combinations. These features of the model are generally supported by developmental data. Representative data from three birds, one tutored with forward-order phrase pairs (48) and two others tutored with reversed-order pairs (67 and 84) are shown in Fig. 5.

Early in song development, birds produced predominantly isolated phrases and phrase pairs; that is, rarely were sequences of more than two phrases produced wherein the gap between consecutive phrases was 200 ms or less (Fig. 5a–c); this conclusion also held for 'acceptance windows' of 0–100 ms and 0–300 ms. At this stage, phrases were placed in a variety of orders (Fig. 5d–f). Over time, correct phrase pairings became more common (compare solid versus dashed lines).

In late plastic song (approximately 200–250 days after the last tutoring session), the decline in phrase pairs and emergence of longer sequences (Fig. 5a–c) reflected the route of song assembly by each bird. Bird 67 produced progressively more triplet sequences (DCB in particular) as the frequency of isolated and paired phrases declined. He constructed his DCBA song by then adding a whistle to the end of the DCB sequence; the appearance of quadruplet sequences in the plot reflected the tightening of the temporal coupling of the DCB and A elements. Bird 48 also produced primarily isolated phrases and phrase pairs early in development, but his assembly of an ABCD song was not preceded by a large increase in triplet sequences. Instead he primarily combined phrase pairs in various orders, eventually constructing his song by tightening the coupling between AB and CD sequences, and eliminating other arrangements; BC pairs, although quite common early in development, were not used in constructing the final ABCD song. Bird 84 assembled his final song (EDBA) by combining ED and BA phrase pairs. In mid-plastic song, the gap between ED-BA phrase pairs was approximately that seen in EDCBA songs, that is, appropriate for a C-type phrase, even though it was rarely expressed. During late plastic song, the emergence of the EDBA sequence followed the tightening of temporal coupling of these phrase pairs. DC pairings, however, were relatively rare, which may explain why this bird did not produce a DCBA song.

We have presented the first evidence that songbirds can assemble complete songs when only tutored with pairs of phrases. Furthermore, they were able to extract the correct sequence of phrases under both conditions of phrase-pair tutoring, forward and reverse. Birds crystallized the forward phrase sequence more successfully, consistent with a synergy between innate (for example, beginning songs with a whistle) and experience-based forces in the forward-order regimen. The conceptual model provides a mechanism by which motor programs for linking particular phrase types together are reinforced on a pair-by-pair basis. The reinforcement is assumed to result from activating, through auditory feedback, particular combination-sensitive neurons, although a direct influence may also be possible¹¹. Recordings in forebrain regions, particularly the HVC, of anaesthetized songbirds have shown that neural mechanisms for generating selectivity for phrase order or phrase combinations exist^{12–17}, although this selectivity emerges only during the sensorimotor phase of song development^{18–20}. Through pair-by-pair reinforcement, the correct sequence emerges as the phrases are generated, possibly by chance, in the correct order. The developmental process of comparing what is produced to the memory (template) of the tutor models may involve interaction between basal ganglia and vocal motor circuits^{1,21}. Similarly, the basal ganglia in mammals are believed to play a part in controlling sequential motor behaviour²², and, as in songbirds, are implicated in vocal learning²³. Thus the song system may be broadly relevant for understanding how the nervous system

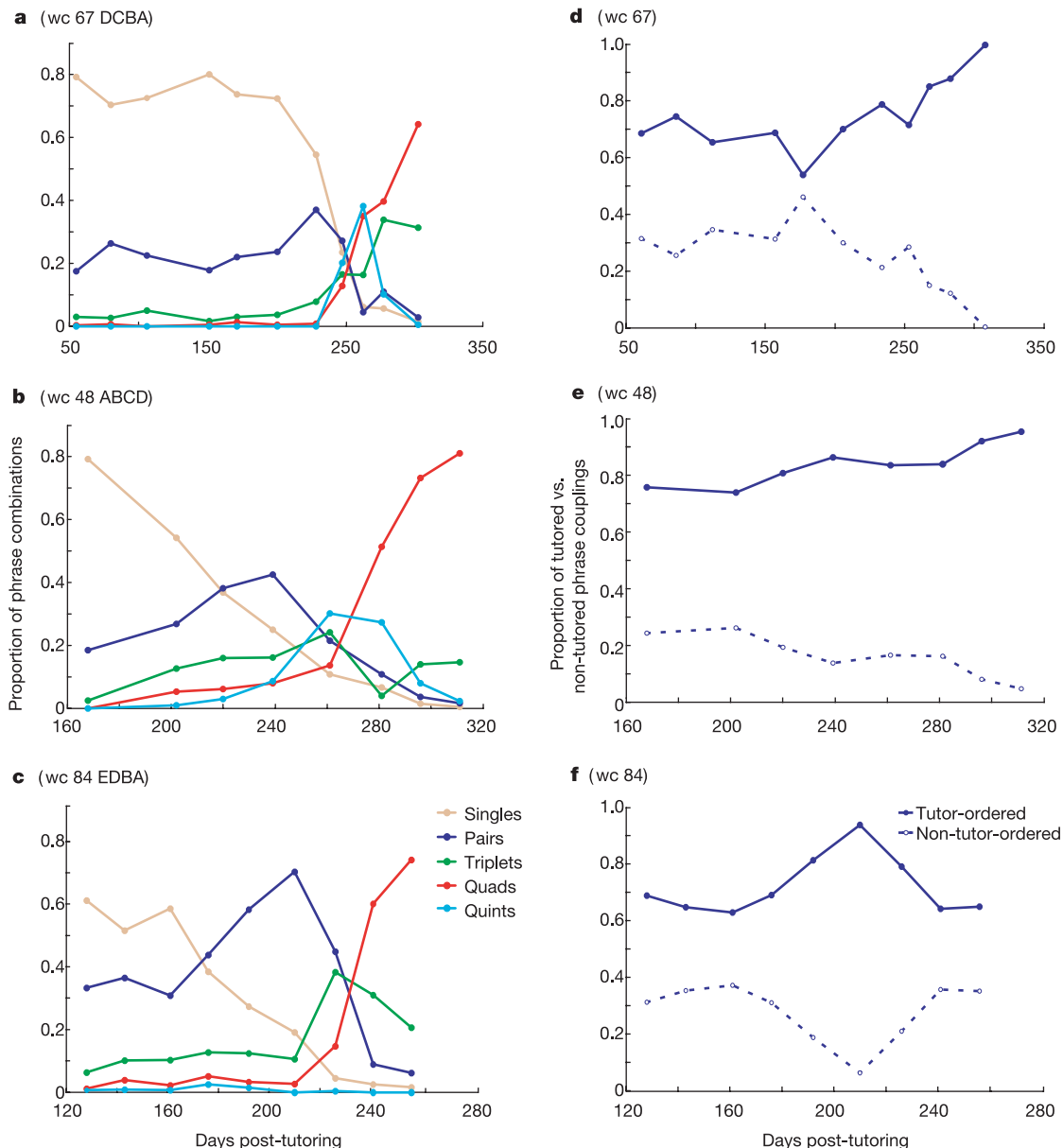


Figure 5 Song development for birds tutored with forward (bird 48) or reversed-order (birds 67 and 84) phrase pairs. **a–c**, Proportion of particular phrase groupings versus number of days elapsed from the last tutoring session; phrases were considered to be grouped if less than 200 ms transpired between the end of one phrase and the start of the subsequent phrase. **e–f**, Proportion of correct (solid lines) and incorrect (dashed lines)

phrase adjacencies over time; data were pooled over 15-day periods, with x-axis values being the first day of each of these periods. The earliest data presented varied across birds because of individual differences in the refinement of syllable and phrase structure.

encodes information about temporally related events and generates motor sequences. □

Methods

Subjects

White-crowned sparrows (*Zonotrichia leucophrys oriantha*) were collected as nestlings (5–7 days after hatching) in Wasatch County Utah during June and July of 2002 and 2003. Birds were hand fed until several days postfledging, and then given seed, puppy-chow, water and an assortment of fresh foods including crickets and mealworms. Fledglings were group-housed, 2–4 birds per cage, and tutored starting at 10–14 days of age.

White-crowned sparrows that have been exposed to song only as nestlings (less than 10 days of age), then raised in acoustic isolation, develop abnormal songs indistinguishable from those produced by 'isolate' birds that were raised from the egg⁴. Hearing song as a nestling is insufficient, therefore, for acquiring a template that can later, through auditory feedback, be used to guide song development.

Tutoring ensembles and procedures

The songs of two white-crowned sparrows (Fig. 1a), recorded at the location where hatchlings were collected, served as the starting material for constructing the tutoring ensembles. These songs were digitized (25 kHz) using Signal (Engineering Design) or SASLab Pro (Avisoft) sound analysis software. Files were then created that consisted of single phrases or pairs of normally adjacent phrases; for example, whistle-note complexes (AB), note complexes-buzz (BC) and buzz-trill (CD) (Fig. 1b). In the second ensemble, the order of phrases in these pairs was reversed from that of the natural order. A silent interval of 2.5 s separated each phrase (single-phrase tutoring experiment) or phrase pair from the preceding or following phrase or pair. The starting phrase or phrase pair for each session was selected randomly, but the order of presentation was always as shown in Fig. 1b, c; for single-phrase tutoring, phrases were presented in backwards order (E, D, C, B, A). These presentation orders were selected to ensure that birds could not use short-term memory to deduce the appropriate order that phrases should be placed. Sequences of phrases or phrase pairs were separated by 13.5 s.

In each tutoring session, the stimuli were broadcast (about 78 dB SPL) for 10 min then a minute of silence was allowed to transpire before starting the next 10-min segment. Two

tutoring sessions, each 90 min, were given each day. Birds that produced soft (subsong) vocalizations during tutoring were banded and transferred to individual custom sound attenuating chambers where tutoring continued until a total of 60 days was reached. Eight birds were tutored with the forward phrase-pair ensemble, eight others received tutoring with the reversed phrase-pair regimen. Eleven birds were tutored with singly presented phrases.

Birds were sexed in the following summer either by microsatellite DNA analysis²⁴ or autopsy; five of the birds in the reversed phrase-order group and seven in the forward phrase-order group were confirmed to be males.

Recording and analysis of vocalizations

The vocalizations of each bird were recorded using a small condenser microphone that was located in each sound-attenuating chamber. Beginning at approximately 30 days after tutoring, recordings were made frequently, with intervals not exceeding 9 days. These signals were digitized, displayed as spectrograms in real time and stored as computer files (Avisoft Recorder). Across renditions, the crystallized songs of birds often varied in length, with the last phrase sometimes not included. Spectrograms of crystallized songs that are displayed in this paper were chosen to show all of the phrase types of typical length found in these songs. Songs were scored with regard to whether adjacent phrases were placed in correct versus incorrect relative position; these values were then evaluated with respect to what would be expected by chance.

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Early motor activity drives spindle bursts in the developing somatosensory cortex

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Sensorimotor coordination emerges early in development. The maturation period is characterized by the establishment of somatotopic cortical maps^{1,2}, the emergence of long-range cortical connections³, heightened experience-dependent plasticity^{4–7} and spontaneous uncoordinated skeletal movement^{8,9}. How these various processes cooperate to allow the somatosensory system to form a three-dimensional representation of the body is not known. In the visual system, interactions between spontaneous network patterns and afferent activity have been suggested to be vital for normal development^{10,11}. Although several intrinsic cortical patterns of correlated neuronal activity have been described in developing somatosensory cortex *in vitro*^{12–14}, the *in vivo* patterns in the critical developmental period and the influence of physiological sensory inputs on these patterns remain unknown. We report here that in the intact somatosensory cortex of the newborn rat *in vivo*, spatially confined spindle bursts represent the first and only organized network pattern. The localized spindles are selectively triggered in a somatotopic manner by spontaneous muscle twitches^{8,9}, motor patterns analogous to human fetal movements^{15,16}. We suggest that the interaction between movement-triggered sensory feedback signals and self-organized spindle oscillations shapes the formation of cortical connections required for sensorimotor coordination.

We examined the nature of early sensory signals and self-generated activity in the primary somatosensory (S1) cortex, using extracellular mapping and patch-clamp recordings in neonatal rats *in vivo* (postnatal days 1–8). In contrast to the adult neocortex¹⁷, activity in the neonatal rat was characterized by intermittent network bursts (mean±s.d. = 0.65±0.15 s), separated by long silent periods (Fig. 1; 7.2±1.93 s; *n* = 19 non-anaesthetized pups). The multiple unit bursts were associated with a single sharp potential or spindle-shape field oscillations (10.8±1.2 Hz). Unitary discharges were rare between these field events (Fig. 1a, d). Sharp potentials occurred in isolation or preceded spindles by 100–200 ms. Simultaneous recording of field events in various S1 cortical layers with silicon probes showed a reversal of both the sharp potential and spindle pattern between superficial and deep layers (Fig. 1f; *n* = 2 pups). Spindle activity was associated with rhythmic multiple unit discharges (Fig. 1b; see Supplementary Figures).

To explore the synaptic mechanisms underlying the generation of S1 network bursts, we employed patch-clamp recordings in urethane-anaesthetized animals (Fig. 2; see Supplementary Figures). Glutamatergic excitatory postsynaptic currents (EPSCs) and GABA (γ -aminobutyric acid) receptor A (GABA_A) receptor-mediated PSCs were separated by using whole-cell recordings in voltage-clamp mode with low-chloride internal solution. The basic parameters of EPSCs and GABA_A-PSCs were similar to that described *in vitro* (Supplementary Table)¹⁸. A characteristic feature of synaptic activity in S1 neurons was the grouping of both EPSCs

and GABA_A-PSCs into bursts, which coincided with the S1 network events, recorded by a nearby extracellular electrode (Fig. 2b, c). Coincidence of synaptic and population activities was also reflected by the robust cross-correlations between the synaptic currents and S1 bursts and spindles (Fig. 2d, e). Charge transfer mediated by glutamatergic EPSCs and GABA_A-PSCs during spindle bursts was 32 ± 7 pC and 71 ± 29 pC, respectively ($n = 10$ cells). Thus, synchronous excitation of S1 neurons during spindle bursts is brought about by synaptic mechanisms involving glutamatergic connections and by GABAergic synapses in keeping with previous *in vitro* studies^{14,19,20}.

On the basis of event duration, frequency, field waveform, cortical depth profiles and cellular correlates, S1 cortical spindles are homologous to sleep spindles or μ rhythm of the adult cortex, suggesting the involvement of the thalamo-cortical network^{17,21–23}. In support of this hypothesis, recordings from the thalamus ($n = 4$ pups) revealed cortical spindle-related multiple unit discharges (see Supplementary Figures). However, besides being more variable in frequency, two features of S1 spindles were fundamentally different from the adult patterns: spindles in the pup were spatially highly confined and consistently associated with motor activity (Fig. 1). Motor patterns during the first week of life are characterized by spontaneous muscle twitches, limb jerks and whole-body startles^{8,9}, initiated by stochastic bursts in the spinal cord network²⁴. Video monitoring in freely moving pups ($n = 4$) showed that most S1 bursts were associated with overt movement, including isolated muscle twitches, limb and whole-body jerks, crawling and sucking.

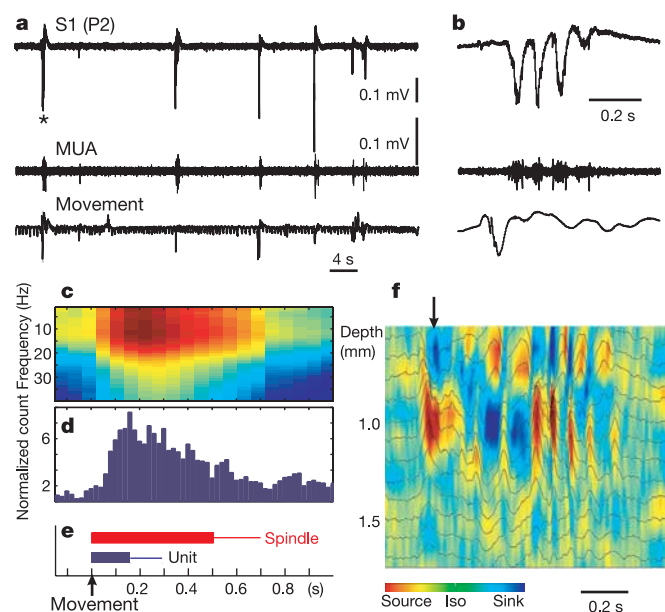


Figure 1 Movement-triggered spindle bursts in S1 of the newborn rat. **a, b**, Wide-band recordings of extracellular activity and filtered (0.3–5 kHz) multiple unit activity (MUA) in S1 hindlimb area of a two-day-old (P2) behaving pup. Positivity is up. Bottom, movement of the contralateral hindlimb. Continuous rhythm reflects respiration. Note that field events and synchronized unit bursts are associated with movements. The event marked by an asterisk in **a** is shown at expanded time scale in **b**. **c, d**, Averaged power spectrogram of field (**c**) and perievent histogram of MUA (**d**), referenced to movement onset (0 s). Note increased power at 5–15 Hz (**c**, normalized colour code) and MUA rate (**d**, normalized spikes per bin) between 50 and 550 ms. The same pup was used in **a–d**. **e**, Mean (+s.d.) delay between movement peak and peak of spindle power, and movement and unit firing ($n = 19$ pups; $P < 0.001$ in each). **f**, Transcortical current source density of sharp potential (arrow) and spindle recorded by a 16-site silicone probe in a P5 pup. Colours indicate changes in current flow in (sink) and out (source) of neurons, relative to an isoelectric (iso) state.

Temporal analysis between mechanically detected movement and S1 activity showed that movement consistently preceded cortical unit bursts and associated sharp potentials and spindles (Fig. 1c–e). Only 14% (+11 s.d.) of all spindles occurred in the absence of overt movement. To investigate the spatial localization of S1 bursts, an array of eight electrodes was placed in the same cortical depth in 15 pups. Spindle bursts, associated with movements of the contralateral forelimb and hindlimb, were spatially confined to different cortical areas (Fig. 3a, b; see Supplementary Figures). After this step, the pups were lightly anaesthetized with urethane (1 g kg^{-1}) and the effect of sensory stimulation was examined. Mechanical touch ($n = 15$) or electrical stimulation ($n = 5$ additional pups) of the limbs and other body parts evoked a spatially confined sensory potential, often followed by a spindle. The magnitude, latency and cortical spatial distribution of the stimulation-evoked potential and spindle power were similar to that of sharp potentials and spindles triggered by the isolated, spontaneous limb movements (Fig. 3; see Supplementary Figures). The evoked field events were associated with multiple unit discharges and collective EPSCs and GABA_A-PSCs (see Supplementary Figures). These results show that S1 spindle bursts are selectively triggered in a somatotopic manner by sensory feedback signals, resulting from spontaneous movements. Their spatial confinement, relative to adult sleep spindles^{17,21,22}, may be explained by poorly developed long-range cortical connections at this early age^{3,25}.

Is sensory input a necessary trigger for cortical spindles? To answer this question, sensory inputs from the hindlimb area were

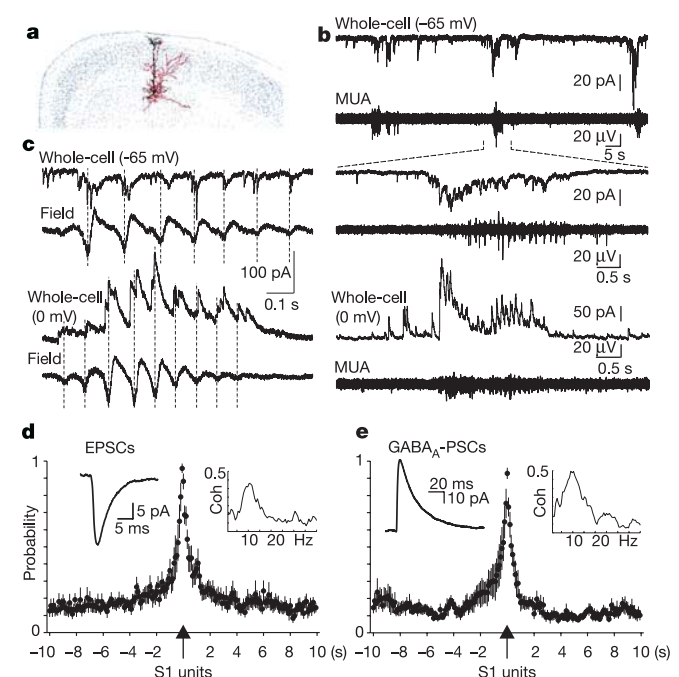


Figure 2 Synaptic correlates of S1 spindle bursts. **a**, Reconstruction of a whole-cell recorded and biocytin-filled layer V pyramidal cell (red, axon; white, dendrites; depth, 800 μm ; P5 pup). **b**, Voltage-clamp recording from the neuron in **a**, at holding potential -65 mV (the reversal potential of the GABA_A-receptor-mediated currents) and concomitant MUA (depth: 900 μm). The middle burst is shown at higher temporal resolution. Bottom traces, GABA_A-PSCs at 0 mV (the reversal potential of the glutamatergic EPSCs) and concomitant MUA. **c**, Same as in **b** but from another cell (P6 pup). **d, e**, Normalized cross-correlograms between S1 MUA and EPSCs (**d**) and S1 MUA and GABA_A-PSCs (**e**). Data represent the mean and s.e.m. from nine cells. Left insets, averaged spontaneous EPSCs and GABA_A-PSCs from the neuron in **a** (see also Supplementary Table S1). Right insets, coherence (coh) spectra between field potential and PSCs for the neuron in **c**. Note high coherence at 10 Hz.

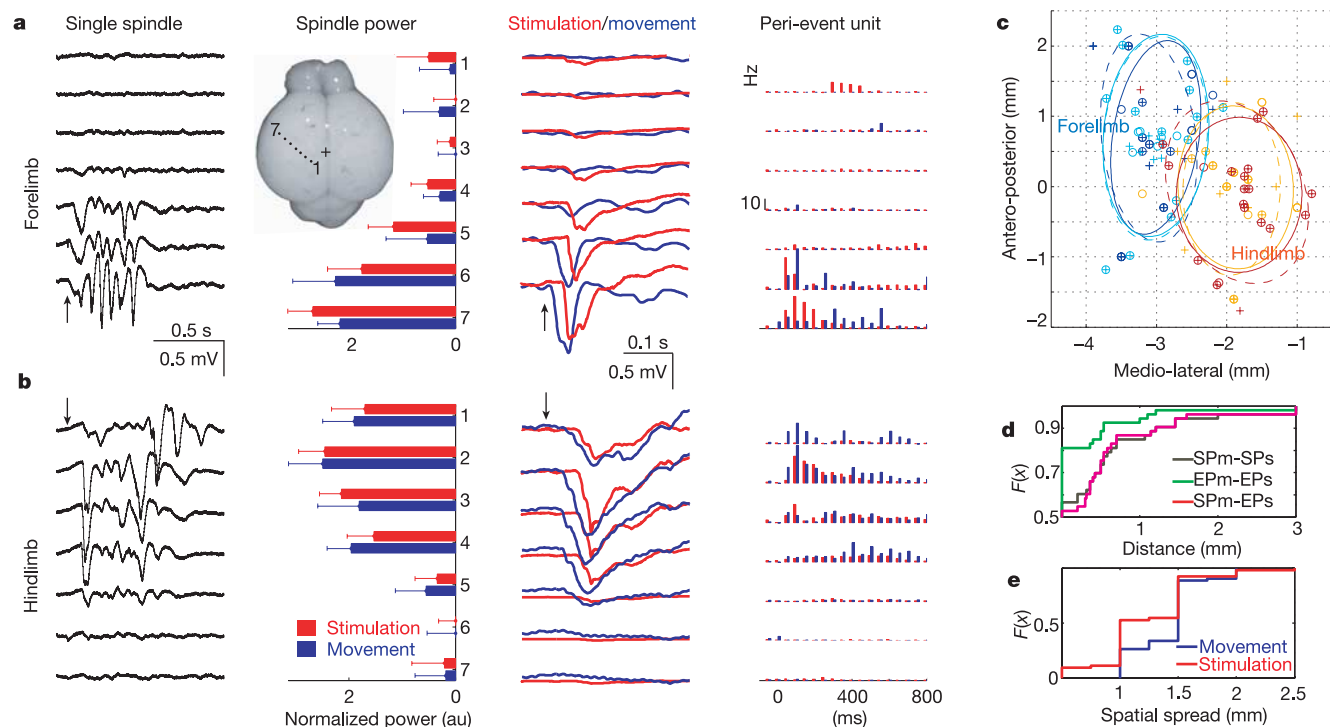


Figure 3 Cortical spindles are spatially confined. **a**, First column: a single spindle event associated with contralateral forelimb jerk (arrow). Second column: power of spindle events triggered by spontaneous forelimb movement ($n = 11$, blue) or by touch stimulation of the forelimb ($n = 34$, red). Inset: position of recording electrodes. +, bregma. Third column: averaged field responses. Fourth column: peri-event time histograms of unit activity triggered by forelimb movement (blue) or touch stimulation (red). **b**, same as in **a**, but related to contralateral hindlimb activity ($n = 20$ movements, $n = 90$ stimulations). **c**, Spatial map (in stereotaxic coordinates) of maximal spindle

power (crosses) and amplitude of field potentials (circles), triggered by movement (blue, red) or evoked by touch (green, orange), referenced to forelimb (blue-green) and hindlimb (red-orange), based on 15 pups. Ellipsoids show 75% confidence intervals. **d**, Cumulative probability density function of distances between locations of maximal spindle power triggered by movement or touch stimulation (SPm-SPs), maximal amplitude of evoked potential by movement or touch (EPM-EPs), maximal spindle power by movement or evoked potential by touch (SPm-EPs). **e**, Cumulative probability density for effective spatial size of movement-triggered and touch stimulation evoked spindles.

disconnected by severing the spinal cord at low-thoracic level after mapping the topography of spontaneous and sensory evoked spindles ($n = 5$ anaesthetized pups). The incidence of spindles in the hindlimb cortical area in the absence of peripheral input decreased threefold, but nevertheless spindles persisted. In the unaffected forelimb area spindle probability was not affected (Fig. 4). These findings provide evidence that spindles are self-organized patterns, generated by circuits that are intrinsic to the brain, but in the neonatal animal the movement-triggered events keep delaying the occurrence of spontaneous events because the rate of jerks is higher than that of the endogenous spindles.

In conclusion, the main pattern of activity in the newborn S1 cortex is a localized network burst, typically organized into a spindle pattern. Spindle bursts are consistently triggered in a somatotopic manner by sensory feedback from the characteristic neonatal muscle twitches, and limb and body jerks. Importantly, human fetal movements *in utero* are similar to the twitching movements in the neonatal rat^{8,15,16} and, according to our findings, may be the main source of sensory inputs to the somatosensory cortex *in utero*. The scalp electroencephalogram (EEG) of preterm infants is characterized by intermittent bursts of electrical activity, often organized into rhythmic patterns ("delta-brush")²⁶, similar to the S1 spindles described here in the rat. Our experiments revealed a link between these two early developmental phenomena, S1 network spindles and spontaneous motor activity. Muscle activity in specific body parts is reliably and consistently associated with discharges of specific neuron groups in S1 cortex. The spatial-temporal coordination of movement (afferent feedback) spindle sequences may serve to anchor body representation in S1 cortex to the physical layout of

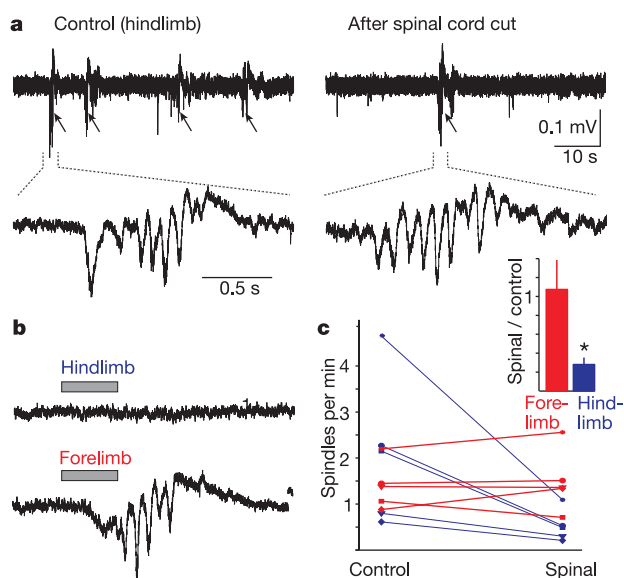


Figure 4 Spindles can occur in the absence of sensory inputs. Effect of low-thoracic spinal cord transection on the neuronal activity in the hindlimb S1 area. **a**, Recordings from the hindlimb area before (left) and 90 min after (right) low-thoracic cut of the spinal cord. Arrows, spindle bursts. Two events are shown below at expanded time scale. **b**, Touch-evoked spindle events continued to occur after spinal transection in response to stimulation of the contralateral forelimb, but not hindlimb. **c**, Incidence of spindle events from five pups (P5–7) in the forelimb and hindlimb area before and after lesion. Bar graphs: mean ($\pm$ s.d.) ratio of the frequency of spindle events before and after lesion ($*P < 0.002$).

the muscular system and provide metric coordinates for sensation. As the initially slow-conducting thalamo-cortical and long-range motor-sensory connections develop^{3,25}, the temporal prolongation of sensory feedback by the self-generated spindle may assist in strengthening the new connections, thus contributing to the establishment of sensorimotor coordination. □

Methods

Neocortical recordings were made from freely moving and anaesthetized neonatal rats (post-natal days (P) 1–8; $n = 45$)²⁷. In freely moving pups ($n = 7$), 4–8 tungsten wires (20 μm in diameter) with 100–300 μm vertical tip separation were implanted at age P4 (–0.5–1.5 mm posterior to the bregma and 1.5–2.0 mm from the midline) under ice-cooling anaesthesia. In 17 additional pups (P1–P6), two anchor bars were fixed to the skull under isoflurane anaesthesia. After recovery, the head was restrained by the skull bars and the body was surrounded by a cotton nest, mimicking the presence of littermates^{28,29}. An array of eight electrodes was placed in the same cortical depth in S1 so that at least some of them recorded units. A water heater placed under the nest kept the body temperature constant at 35 °C. Acute recordings ($n = 21$) were performed under urethane anaesthesia (1–1.5 g kg^{–1}). For simultaneous recording of field potential and multi-unit activity, silicon probes³⁰ or tungsten wires (20 μm in diameter) were used. The silicon probe (16 recording sites with 100 μm separation) was inserted vertically into the brain so that simultaneous recordings could be made from the somato-sensory cortex at various depths. Whole-cell patch-clamp recordings were performed in the vicinity of the extracellular electrode ($n = 9$ rats)²⁷. Morphological identification of the recorded cells was performed by adding biocytine (0.4%) to the pipette solution. Pyramidal cells with typical apical dendrite ramifying close to the brain surface were found in layer V ($n = 4$). Data were acquired at a sampling rate of 20 kHz and analysed off-line. Oscillatory events were detected by filtering the raw data in respective frequency ranges, identifying periods of high power of sufficient duration and then detecting the troughs. Multi-unit bursts were detected from the discriminated spike trains. Movements were recorded at 30 frames per second and reviewed frame-by-frame. The beginning and the end of all motor movements, including phasic and tonic, local and generalized movements were detected by eye. For finer temporal resolution, movements of the forelimb, hindlimb and body were detected by miniature earphones placed on the limbs. Sensitivity was sufficient to detect the smallest visible movements, including respiration-related displacement of the limbs (Fig. 1). Further details are provided in the Supplementary Methods.

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A physical map of the chicken genome

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Strategies for assembling large, complex genomes have evolved to include a combination of whole-genome shotgun sequencing and hierarchical map-assisted sequencing^{1,2}. Whole-genome maps of all types can aid genome assemblies, generally starting with low-resolution cytogenetic maps and ending with the highest

resolution of sequence. Fingerprint clone maps are based upon complete restriction enzyme digests of clones representative of the target genome, and ultimately comprise a near-contiguous path of clones across the genome. Such clone-based maps are used to validate sequence assembly order, supply long-range linking information for assembled sequences, anchor sequences to the genetic map and provide templates for closing gaps. Fingerprint maps are also a critical resource for subsequent functional genomic studies, because they provide a redundant and ordered sampling of the genome with clones³. In an accompanying paper⁴ we describe the draft genome sequence of the chicken, *Gallus gallus*, the first species sequenced that is both a model organism and a global food source. Here we present a clone-based physical map of the chicken genome at 20-fold coverage, containing 260 contigs of overlapping clones. This map represents approximately 91% of the chicken genome and enables identification of chicken clones aligned to positions in other sequenced genomes.

For large genome sequence assemblies so far, contiguous clone-based maps provide the framework for organizing the sequence^{5,6}. With the increasing emphasis on whole-genome shotgun assemblies, integration of mapping data throughout the assembly and finishing process is key to improving efficiency and assuring accuracy equivalent to human genome standards. The basis of map construction for any genome is the reliance on random breakage of genome structure and the subsequent ordering of these genome pieces. Once assembled, physical maps are a key resource in the dissemination of clones by known location for use in all disciplines of biology.

A large number of chicken genomic resources are available, including a collection of large-insert bacterial artificial chromosome (BAC) clone libraries^{7,8}. These libraries were made from the inbred red jungle fowl 256 (RJF, *G. gallus*) strain used in whole-genome sequencing and from the white leghorn, a domestic breed selected for egg production. Meiotic linkage maps for three mapping populations were used to develop a consensus map containing about 2,000 sequence-tagged site (STS) markers^{9,10}. Markers that were assigned to BACs were a key resource in map construction.

We constructed a physical clone map from 154,560 fingerprints of *G. gallus* BACs. Automated band recognition software identified restriction digest fragments¹¹. A small fraction (15%) of these fingerprints were lost due to empty lanes, empty insert clones, incomplete restriction enzyme digestion, or failure by the software to recognize lanes. The FPC program processed the remaining 130,486 fingerprints¹². Automated overlap evaluation of these fingerprints generated 6,509 contigs using a Sulston score¹³ threshold of 1×10^{-17} , tolerance of 7. The Sulston score approximates the probability of two clones realizing a given number of fingerprint band matches by coincidence. These parameters left 51,954 singleton clones not assigned to a contig. The final automated step refined clone order using the program CORAL¹⁴. CORAL has an improved clone-ordering algorithm proven effective when applied to individual FPC contigs.

Subsequent to automated map assembly, manual review is an essential step in building a reliable fingerprint map. Although a required element in high-throughput generation of fingerprints, automated identification of bands produces band-calling errors that cannot be completely eliminated. These errors propagate during clone ordering and result in incorrectly assembled map contigs. Dispersed and tandem sequence repeats can also confound software programs, collapsing different regions into a complex mix of falsely overlapping clones. Finally, there is currently no automated procedure for merging related contigs. Therefore, we visually examined the fingerprint images of digested clones in each contig to limit errors in automated contig construction. Clone order errors were resolved and fingerprints not meeting expectations for band pattern

were removed from contigs and returned to the singleton pool. To merge overlapping contigs, we relaxed the Sulston score threshold to 1×10^{-10} and then to 1×10^{-7} . Clones comprising the terminal ends of contigs were allowed to join other contigs after manual review. We added individual singleton clones to contigs as needed to increase coverage of sparse regions. If a clone did not provide further band information, it was left in the singleton pool. Of the initial 6,509 contigs, 95% were joined to produce 320 contigs.

Markers are essential for anchoring the fingerprint map to chromosomes, and they also permit validation of contig integrity. A limited number ($n = 911$) of STS markers have been mapped to fingerprinted RJF clones; 730 of these have chromosome placements¹⁵. The white leghorn fingerprint database, however, is a rich source of marker data, with 1,830 markers (1,717 assigned to chromosomes). To increase the number of links to the genetic map, we added the fingerprints of 49,805 white leghorn BAC clones to the physical map. We assigned 29,663 white leghorn clones to contigs, based on the restrictive condition that each of these clones had a fingerprint match to at least five RJF contig clones. Manual inspection confirmed the positions of 2,244 white leghorn clones having assigned markers. The white leghorn clones were used for their marker information only; their fingerprints were not used to join RJF contigs.

We also took advantage of the draft sequence assembly to refine further the fingerprint map. The assembly and fingerprint map are linked by 128,523 BAC-end sequences (BES). After requiring a minimum of six end-sequence links, a total of 189 fingerprint contigs were reliably assigned to the sequence assembly. Some of the contigs left unassigned were linked to assembly contigs in a topologically impossible manner, indicating errors in the assembly or fingerprint map. For example, a pair of contigs cannot be correctly linked by their ends if the middle portions are linked to different contigs. Potentially incorrect regions of the assembly and fingerprint maps were reciprocally examined, revealing 14 mis-assembled fingerprint contigs that were subsequently split apart. These cases were primarily due to contaminated clones missed during the manual review process.

The assembly also provided preliminary order to 3,557 singleton clones not assigned to fingerprint contigs. Small groups of overlapping singletons often served as bridges that could join contigs. Merges suggested by the assembly were examined and made only when supported by fingerprint data, although a Sulston score of 1×10^{-6} was accepted with the additional sequence evidence. A summary of clone distribution in the final 260 contigs is shown in Table 1.

The genetic map was then used to anchor the fingerprint contigs to chromosomes. We determined the distribution of contigs on each chicken chromosome using a simple plurality of chromosome assignments for the marker-clone pairs in each contig. Markers assigned to white leghorn clones were not considered unless the contig location of the clone had been manually confirmed. A total of

Table 1 Summary of clone distribution in the physical map

Number of clones	180,291
Number of singletons	47,070
Number of contigs	260
Contig size distribution	
> 200 clones	105
101–200 clones	32
51–100 clones	33
26–50 clones	32
10–25 clones	35
3–9 clones	22
2 clones	1

Singletons include 19,617 white leghorn and 27,453 RJF clones.

186 contigs were assigned in this way. Linkage of the fingerprint map to the sequence assembly provides positional information for many contigs. All 125 contigs suggested by the assembly to be collinear had consistent chromosome assignments. An additional seven contigs lacking independent marker data were given chromosome assignments based on their linkage to an assigned FPC contig. Finally, markers positioned on assembly contigs by BLAST sequence comparison provided additional support of the chromosome assignments and allowed localization of an additional 33 fingerprint contigs based on their linkage to assembled sequence. A summary of the 226 contigs mapped to chromosomes is found in Table 2.

A minimally overlapping set of clones spanning the map contigs is useful for comparative genomic studies¹⁶, as a source of specific cloned sequence, and as a reliable estimator of the physical length of each contig. We used the software Minilda (<http://mkweb.bcgsc.ca/minilda/>) to select clones with the goals of maximizing the amount of unique content in each clone selection, limiting excessive overlap, avoiding gaps between adjacent selections, and avoiding clones with fingerprint bands unconfirmed by overlapping clones. Our estimated minimum tiling path set consists of 9,210 BAC clones with an average clone overlap of 77 kilobases (kb) (<http://mkweb.bcgsc.ca/chicken/images/?list=003>). Estimates of the physical size of each contig were made and the totals per chromosome are listed in Table 2. The total amount of sequence represented in fingerprint contigs is 0.97 gigabases (Gb), or 91% of the current sequence assembly⁴.

Low coverage BAC-based fingerprint maps of the chicken genome were recently published^{7,8}. The maps represent preliminary efforts to generate resources for the community, such as region-specific BAC clones as templates for polymorphic marker development. Ren *et al.*⁷ report 2,331 contigs, estimated to cover $\sim 7.5 \times$

genome equivalents, with 367 markers used to anchor only 11% of the contigs. The estimated $3.6 \times$ genome coverage by Aerts *et al.*⁸ was insufficient for accurate comparisons of clone distribution by contig. Both of these reports represented progress towards developing clone resources for functional genomic studies and for improving knowledge of local clone order for selected chromosomes; for example, *G. gallus* chromosome 10 (GGA10)⁸. However, neither report created the comprehensive coverage required for sequence assembly validation or provided a sufficient resource to pick tiling paths of reduced genome representation. Our manually curated map of 180,291 clones and 260 contigs represents a major advance upon these early efforts, while at the same time incorporating the identical libraries. In particular, this map integrates an additional, much larger BAC library (CHORI-261), 2,628 STS markers, 128,523 BAC-end sequences, the chicken whole-genome sequence assembly, and much higher clone redundancy. These additional resources allowed us to improve greatly clone contig distribution as demonstrated by a 100-fold increase in large contigs, defined as >200 clones per contig (see Table 1). The physical map is available in standard formats, such as CMAP (<http://gmod.wustl.edu/cgi-bin/cmap/viewer>) and GBrowse (<http://www.animalsciences.nl/ChickFPC>). It can also be downloaded directly from http://genome.wustl.edu/pub/groups/mapping/fpc_files/chicken/.

Combining physical maps with a whole-genome, shotgun-based strategy provides an ideal blueprint for sequence assembly¹⁷. Critically, the physical map with corresponding marker information allowed reciprocal error checking of the physical map and the whole-genome shotgun assembly while at the same time providing invaluable long-range linking information. This allowed anchoring, ordering and orienting the sequence along the chicken genome. The clone-based map now also provides a key resource for moving towards improving the chicken genome sequence, filling gaps and sorting out difficult regions of the chicken genome. Future avian molecular genetic research will be greatly aided by this BAC-based map and accompanying minimum tiling path. □

Table 2 Summary statistics of chicken clone map coverage by chromosome

Chicken chromosome	Chromosome size (cM)	Sequenced chromosome size (Mb)	Total fingerprint contigs	Fingerprint contig coverage (Mb)
GGA1	553	188.2	29	188.8
GGA2	474	147.6	18	146.9
GGA3	317	108.6	19	107.8
GGA4	270	90.6	8	88.7
GGA5	199	56.3	10	53.1
GGA6	116	33.9	2	34.1
GGA7	165	37.3	5	32.6
GGA8	105	30	3	26.1
GGA9	132	23.4	6	23.8
GGA10	120	20.9	4	19.7
GGA11	90	19	3	20.0
GGA12	90	19.8	5	18.1
GGA13	74	17.3	4	18.1
GGA14	77	20.6	8	18.1
GGA15	60	12.4	3	12.4
GGA16	60	0.2	2	1.1
GGA17	70	19.6	4	10.1
GGA18	48	8.9	3	8.9
GGA19	41	9.5	3	10.0
GGA20	62	13.5	4	13.7
GGA21	70	6.2	3	6.0
GGA22	21	2.2	3	3.2
GGA23	11	5.7	3	5.3
GGA24	60	5.9	3	5.8
GGA26	54	4.3	2	4.0
GGA27	60	2.7	5	3.5
GGA28	74	4.7	8	6.1
GGA32	21	1	1	0.4
GGAW	NE	0.15	5	2.8
GGAZ	100	31	45	60.8
E22C19W28	NE	0.07	1	0.5
E26C13	52	0.23	2	0.7
E50C23	41	0.02	2	0.7
Unlocalized	NE	121	34	13.1
Total	3,687	1,063	260	964.9

NE, not estimated.

Methods

Fingerprinting

Isolation of plasmid DNA from individual BAC clones was performed according to the manufacturer's recommendations (Brinkman Instruments). The *Hind*III digestion and agarose gel fractionation of BAC clones from TAM31, TAM32 and TAM33 (ref. 7) (<http://hbz.tamu.edu/bacindex.html>), CHORI-261 (<http://bacpac.chori.org>) and WAG⁸ (<http://www.zod.wau.nl/abg/>) libraries were as described previously¹⁸. After fluorescent staining and imaging, gel images were analysed using IMAGE software¹⁹ (<http://www.sanger.ac.uk/Software/Image>), followed by automated band calling with BandLeader¹¹.

BAC end sequencing

End sequences of BAC plasmids were obtained using previously established methods with minor modifications²⁰. Resulting traces were transferred into a database and processed using Autobacend software (S.L., unpublished). All trace files were submitted to the NCBI trace archive (<http://www.ncbi.nlm.nih.gov/Traces>).

Map construction

Fingerprints were assembled into contigs using FPC¹² with a Sulston score threshold of 1×10^{-17} and tolerance of 7. CORAL was used to improve clone order after automated contig builds using FPC. During the manual review of clone order, fingerprint band patterns were compared across a contig. A given band should be found in all correctly positioned clones from a limited region, allowing one to scan a contig from one end to the other, confirming the presence or absence of bands in adjacent clones. An incorrectly positioned clone breaks the overall continuity of banding pattern and was repositioned or removed from the contig. Occasionally, a lane had the fingerprint patterns of two unrelated clones. Clone contamination was confirmed by searching the FPC database for the clone with a similar library name and an exact subset of bands. In this way 576 contaminated clones were removed. A limited number of partially digested clones were also removed from contigs. It is particularly important to order correctly the clones at contig ends. Overlapping contigs are identified by comparing the fingerprints of clones at their ends using successively relaxed Sulston score thresholds. Clones are added from the singleton pool as needed to increase coverage in junction regions when contigs are merged. No attempt was made to add all singleton clones to contigs.

Addition of white leghorn clones

White leghorn clones did not integrate well into contigs made from the larger RJF clones owing to small insert sizes. The white leghorn clones tended to form separate contigs,

largely because the Sulston score takes into account total band number as well as the number of bands matching between two clones. Consequently, white leghorn clones were added to the map based only on their fingerprint matches to RJF clones. The criteria for adding white leghorn clones to RJF contigs were Sulston score threshold and number of RJF contig clones matching at that threshold. We examined the number of marker position inconsistencies introduced after adding white leghorn clones to choose appropriate parameters. The heuristically determined requirement of fingerprint matches to a minimum of five RJF contig clones at a Sulston score threshold of 1×10^{-6} limited marker inconsistencies yet added a significant number of white leghorn clones with positional information to contigs. Manual comparisons of white leghorn fingerprints to surrounding clones were limited to the clones that have assigned STS markers.

Fingerprint map linkage to the draft sequence assembly

The threshold of BES links used to declare regions of the fingerprint map and draft sequence collinear is subjective. Our choice of parameters was guided by their impact on the frequency of marker inconsistencies and number of topologically impossible contig combinations. A minimum of six BES links were required before aligning an FPC contig to a sequenced region, provided that 70% of all links were consistent and no topological constraints were violated. With these parameters, the overall frequency of spurious BES links from an FPC contig to non-collinear sequence contigs was 0.10 and the frequency of BES links to sequence contigs that were not aligned to the FPC map was 0.05. Discrepant BES links reveal regions of the FPC map where manual review might be useful. Each FPC contig was scanned using a sliding window of ten BES links, which corresponds to roughly 109 kb (assuming a genome size of 1.06 Gb and a random distribution of BES links). If four or more BES links were to an unaligned sequence contig, that FPC region was flagged for review. Of the 195 flagged regions, 14 contained contaminated clones. The remaining number could be a reflection of the reliability of BES links, errors in fingerprint map not revealed by fingerprints, or errors in the sequence assembly.

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Early blastomere determines embryo proliferation and caste fate in a polyembryonic wasp

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Polyembryonic development is a unique mode of metazoan development in which a single zygote generates multiple embryos by clonal proliferation¹. The polyembryonic parasitic insect *Copidosoma floridanum* shows one of the most extreme cases of polyembryony, producing up to 2,000 embryos from a single egg. In addition, this wasp exhibits an unusual polyphenism, producing two morphologically distinct larval castes, termed precocious and reproductive, that develop clonally from the same zygote². This form of development seems incompatible with a model of insect development in which maternal pre-patterning of the egg specifies embryonic axial polarity³. Here we show that maternal pre-patterning in the form of germ plasm creates cellular asymmetry at the four-cell stage embryo of *Copidosoma* that is perpetuated throughout development. Laser ablations of cells show that the cell inheriting the germ plasm regulates both the fate and proliferation of the reproductive caste. Thus, we have uncovered a new mechanism of caste specification, mediated by the regulatory capacity of a single cell. This study shows that the evolution of mammalian-like regulative development of an insect embryo relies on a novel cellular context that might ultimately enhance developmental plasticity.

Polyphenism is a phylogenetically widespread feature of some plants and animals, in which organisms with complex life histories form different phenotypes from a single genome⁴. Because model organisms do not exhibit polyphenism, the developmental basis of this marked genetic plasticity remains largely undetermined. Traditional polyphenic eusocial species such as ants and bees produce reproductive (queen) and sterile (worker) castes from the same genome when separate zygotes are exposed to different hormonal or environmental influences⁵. However, one of the most extreme examples of polyphenism coupled with a complex life history strategy is the clonal production of two different morphs from the same zygote in the polyembryonic parasitic wasp *Copidosoma floridanum*.

Copidosoma parasitizes eggs of the moth *Trichoplusia ni* (Fig. 1a). In contrast with other insects that undergo syncytial early cleavages in which nuclear divisions do not follow the division of cytoplasm⁶, the *Copidosoma* egg undergoes total cleavage forming individual cells⁷. During the first two days, the wasp embryo emerges from its chorion and forms a primary morula surrounded by the extra-embryonic membrane derived from the polar body region (Fig. 1a, grey)⁸. The primary morula then undergoes a series of embryonic movements to 'implant' itself into the developing host embryo (Supplementary movie). After parasitization, the host larva emerges and undergoes five larval instars. During the first to third host larval instars, *Copidosoma* enters the proliferative phase generating many proliferative morulae, each containing thousands of round and apparently non-differentiated cells⁸. This phase represents the developmental novelty initiating the clonal production of up to 2,000 embryos. In the proliferative phase the developmental programme bifurcates to produce two terminal larval phenotypes. During the first to third larval instars some of the morulae initiate

morphogenesis, producing sequentially up to 100 long, slender larvae with strong mandibles, called precocious larvae. These larvae have a complex parasitic function, performing workers' tasks including a defensive role against interspecific competitors⁹ and altering the brood sex ratio¹⁰. The precocious larvae never moult, and die when their reproductive siblings consume the host. The reminder of the proliferating morulae synchronously initiate the *de novo* formation of up to 2,000 embryonic axes in the fourth instar, ultimately producing reproductive embryos^{8,11}. These embryos develop into short, compact larvae that consume the host, moult, pupate and form adult wasps.

Animal embryos studied up to now specify their embryonic axes at the beginning of development¹². In contrast, *de novo* specification of thousands of embryonic axes in *Copidosoma* late in development challenges the current model of early axial specification in metazoans. *Drosophila* is an example of animal development in which extensive axial pre-patterning relies on maternal determinants supplied in the form of specialized cytoplasm containing information directing anterior, posterior and germline development¹³.

At the other end of the continuum is the mouse egg, in which neither a pre-pattern nor examples of specialized cytoplasm such as germ plasm have been demonstrated¹⁴. Mathematical models indicate that it is implausible that maternal pre-patterning has a role in *Copidosoma* development, because maintenance of pre-pattern would be impossible during the extensive proliferative growth³. However, in 1906 Silvestri described a specific region of egg cytoplasm called the oosome that he believed to represent the germ plasm¹⁵. We determined previously that at the four-cell stage, the smallest cell that inherits the oosome becomes dye-uncoupled from other cells⁷ and could be stained with anti-*Drosophila* Vasa antibody¹¹, corroborating Silvestri's proposal that early asymmetries might exist in the *Copidosoma* embryo. To determine the role of pre-patterning in polyembryonic insect embryogenesis we established a molecular marker for the oosome during *Copidosoma* embryogenesis and developed a transplantation protocol that allowed us to map the fate of the oosome-containing cell of the early embryo with the use of laser ablation.

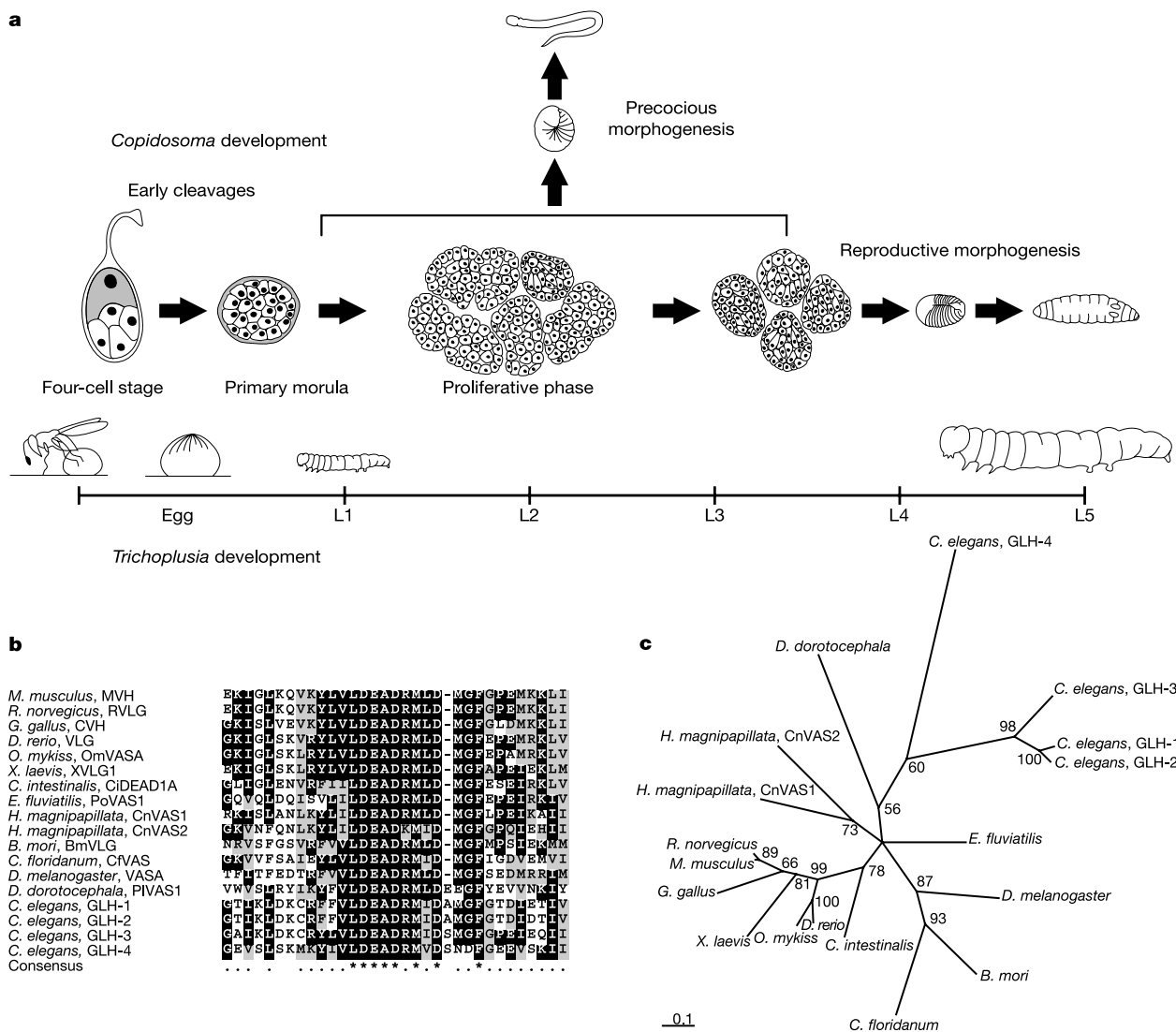


Figure 1 Life cycle of *Copidosoma*, alignment and phylogram of Cfvasa amino acid sequences. **a**, *Copidosoma* life cycle. **b**, Predicted amino acid sequences and alignment of DEAD-box region of *C. floridanum*, *C. elegans*, *H. magnipapillata*, *E. fluviatilis*, *D. dorocephala*, *D. melanogaster*, *B. mori*, *C. intestinalis*, *D. rerio*, *O. mykiss*, *X. laevis*,

G. gallus, *M. musculus* and *R. norvegicus* Vasa proteins; for complete sequence alignment see Supplementary Information. **c**, Phylogram of Vasa amino acid sequences. Numbers at branches represent bootstrap values. Branch-length scale bar represents 0.1 amino acid substitutions per site.

We cloned *Copidosoma vasa* (*Cfvasa*) mRNA, a ubiquitous germline marker in metazoans¹⁶ (Fig. 1b). Phylogenetic analysis of *CfVasa* protein (Fig. 1c) indicates that it clusters with other insect VASA homologues, representing a VASA orthologue in *Copidosoma*. *Cfvasa* mRNA was expressed in nurse cells of early ovarian eggs

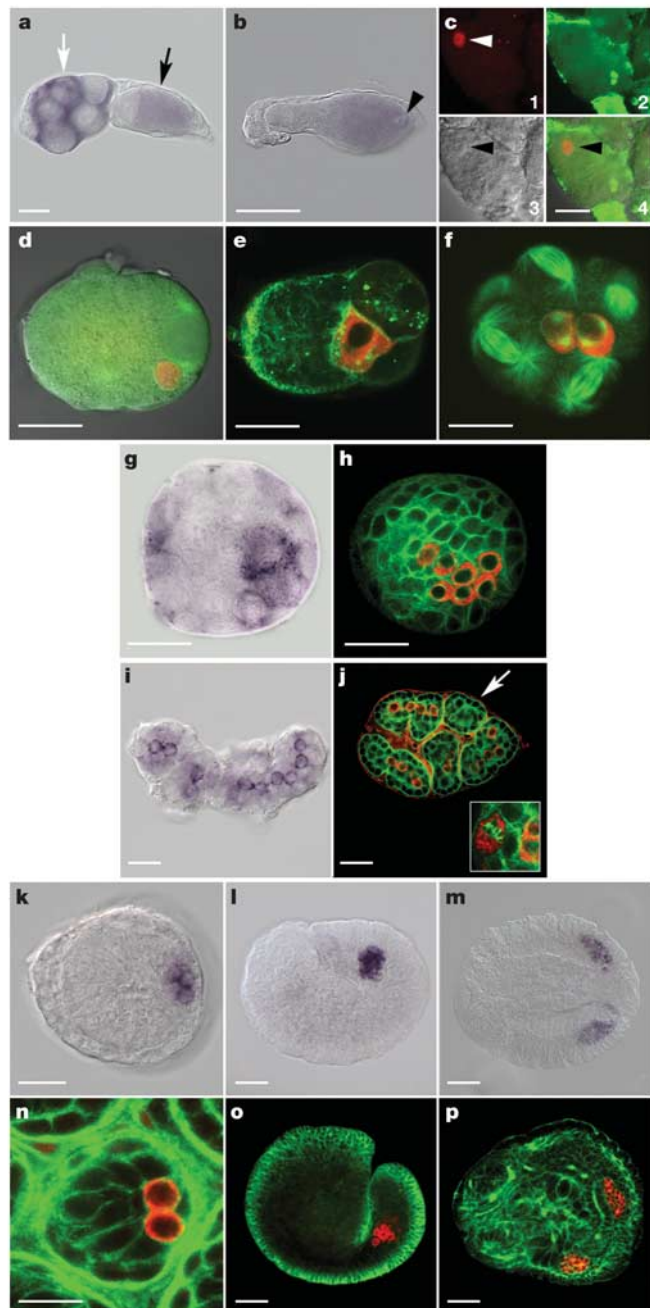


Figure 2 *Cfvasa* mRNA and protein expression patterns during *Copidosoma* embryogenesis. Purple, *Cfvasa* mRNA; red, Vasa protein; green, tubulin. **a–c**, Ovarian eggs. **a**, Early ovarian egg; white arrow points to nurse cells and black arrow points to oocyte. **b**, Late ovarian egg; black arrowhead points to oosome. **c**, 1, Vasa protein expression; 2, tubulin; 3, transmission differential interference contrast image, with arrowhead pointing to oosome; 4, merged image. **d–f**, Early cleavages. **d**, Uncleaved egg. **e**, Second cleavage. **f**, Third cleavage. **g, h**, Primary morula. **i, j**, Proliferative phase. Inset: cleavage of Vasa-positive cell; arrow points to the proliferative morula without germ cells. **k–p**, Reproductive morphogenesis. **k, n**, Early embryonic primordium. **l, o**, Extended germband stage. **m, p**, Completely formed larva. Scale bar 25 μm.

(Fig. 2a). In late ovarian eggs where unique cytoplasm (oosome) is visible (Fig. 2b, arrowhead), unlocalized *Cfvasa* mRNA was detected in the egg chamber. However, staining with anti-Vasa antibody co-localized to the oosome (Fig. 2c), indicating that *CfVasa* protein is a component of the oosome. In the uncleaved egg, *CfVasa* staining continued to mark the oosome that becomes positioned on one side of the spindle apparatus (Fig. 2d). The oosome was then inherited by one large cell in the two-cell stage (not shown) and sequestered invariably to a small cell that stains with anti-Vasa antibody at the four-cell stage (Fig. 2e). The small cell undergoes equal cleavage; both daughter cells inherit an antigen recognized by anti-Vasa antibody (Fig. 2f) and exhibit delayed cleavage relative to the progeny of large cells. In the primary morula, six to eight cells express *Cfvasa* mRNA and protein (Fig. 2g, h). During the proliferative period, Vasa-positive cells are distributed in individual proliferative morulae together with Vasa-negative cells (Fig. 2i, j). Both daughters of Vasa-positive cells inherit Vasa protein during the proliferative period (Fig. 2j, inset). However, some proliferative morulae do not contain Vasa-positive cells (Fig. 2j, arrow). At the onset of reproductive morphogenesis, two Vasa-positive cells are parcelled to each individual embryonic primordium together with about 20–30 Vasa-negative cells (Fig. 2k, n). These cells remain at the posterior of the embryos undergoing morphogenesis (Fig. 2l, o) and become incorporated into paired gonads (Fig. 2m, p). Thus, the distribution of *Cfvasa* mRNA and protein demonstrates that the small cell at the four-cell stage that inherits the oosome is continuous with the germ cells of reproductive larvae. This suggests that the unique cytoplasm forming the oosome represents germ plasm, and that the *Copidosoma* egg exhibits a maternally specified cellular asymmetry that is maintained throughout the proliferative period.

Caste formation in eusocial hymenoptera is a complex series of events in which individual larvae or embryos develop towards the reproductive or sterile caste fate in response to environmental stimuli⁴. However, both sterile and reproductive castes initiate the formation of gonads, which later degenerate in sterile workers and soldiers¹⁷. Even though precocious *Copidosoma* larvae never mature, it has been unclear whether they represent a true sterile morph or whether they simply do not complete their developmental programme because of their premature death. Both *Cfvasa* mRNA and Vasa protein were absent during the course of precocious larva morphogenesis (more than 600 embryos analysed; Fig. 3) even though Vasa protein was expressed in adjacent reproductive

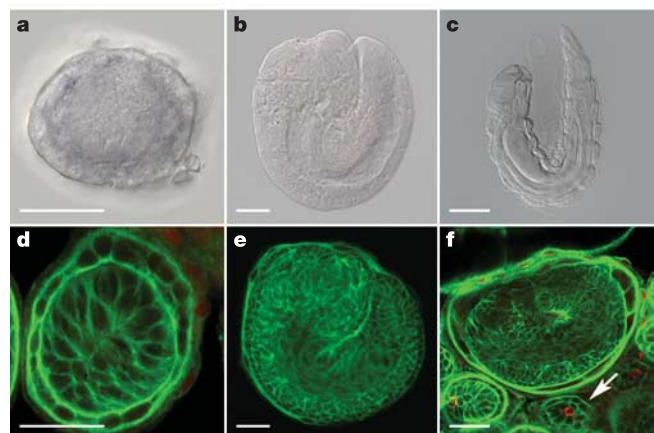


Figure 3 *Cfvasa* mRNA and protein expression patterns during *Copidosoma* precocious larvae morphogenesis (purple, mRNA; red, Vasa protein; green, tubulin). **a, d**, Early embryonic primordium. **b, e**, Extended germband stage. **c, f**, Completely formed larva, arrow points to reproductive embryo with germ cells. Scale bar, 30 μm.

embryos (Fig. 3f, arrow). Thus, precocious morphs originate from morulae that do not contain Vasa-positive cells. The lack of germ cells suggests that precocious morphs represent true sterile morphs and that the distinction between reproductive and precocious morphs is determined by a differential distribution of germline precursor cells.

Because of extensive pre-patterning in many invertebrate eggs, the ablation of early cells or removal of special cytoplasm containing localized determinants results in significant developmental defects¹². In contrast, mammalian embryos show extensive regulative abilities, attributed to a lack of pre-patterning where the removal of early cells results in the formation of normal embryos¹⁸. To determine the role of the Vasa-positive cell in the embryonic development of *Copidosoma*, we laser-ablated cells at the four-cell stage and analysed the development of sham-treated and manipulated embryos (Fig. 4a). To exclude the possibility that embryos fail to initiate development after our treatments, we first cultured subpopulations of embryos *in vitro* to confirm normal development and to assess Vasa expression. Short-term cultured embryos that were sham-treated, Vasa-positive cells that were ablated, and embryos with ablated large cells (Fig. 4b–d) all emerged from the chorion and formed primary morulae, indicating that our treatments did not disrupt normal development. Whereas sham-treated and large-cell ablated embryos showed staining of an expected number of Vasa-expressing cells in the morula stage, no cells showing Vasa staining were observed in Vasa-positive-cell ablated embryos, showing that laser ablation eliminates the progeny of the Vasa-positive cell. In addition, the sham-treated embryos transplanted into a host egg developed along a normal timeline, producing reproductive and precocious larvae (Fig. 4e) in

proportions and numbers almost identical to those of wasps ovipositing naturally¹⁰. These embryos developed into viable adult wasps (not shown), indicating that our transplantation protocol recapitulates the normal life cycle of the parasite.

Germ cells specified by the maternally preformed germ plasm in *Drosophila* and some crustaceans show lineage restriction, exclusively forming the germ line^{19,20}. Consequently, the removal of germ-cell precursors results in a cell-autonomous defect: failure to form gonads. However, mouse and urodele amphibians lack preformed germ plasm, and germ cells are induced later in development in a pluripotent cell population that can give rise to both primordial germ cells and somatic cells^{14,21}. If a Vasa-positive cell is lineage restricted to reproductive embryos in *Copidosoma*, as indicated by our antibody staining, it could have a cell-autonomous role in specifying the germ line. Thus, its ablation would result in the production of both precocious and germ-cell-deficient reproductive larvae. Alternatively, a cell with early asymmetry could impose organizational properties on the embryo¹², exerting non-cell-autonomous influences. In this model, cell ablation should cause broader effects, not limited to the germ line. Dissection and counting of larval morphs at the fifth host instar revealed that total embryo proliferation decreased by almost 95% in embryos in which the Vasa-positive cell was ablated at the four-cell stage. The population of larvae dissected from the host consisted exclusively of precocious morphs in numbers similar to those found in the sham-treated embryos ($t = 0.24$; $P = 0.81$). This indicates that the Vasa-positive cell might have a role in the development of reproductive embryos, by regulating their proliferation and specifying reproductive caste identity. To test whether this is a unique property of the Vasa-positive cell, we individually ablated the other three large cells.

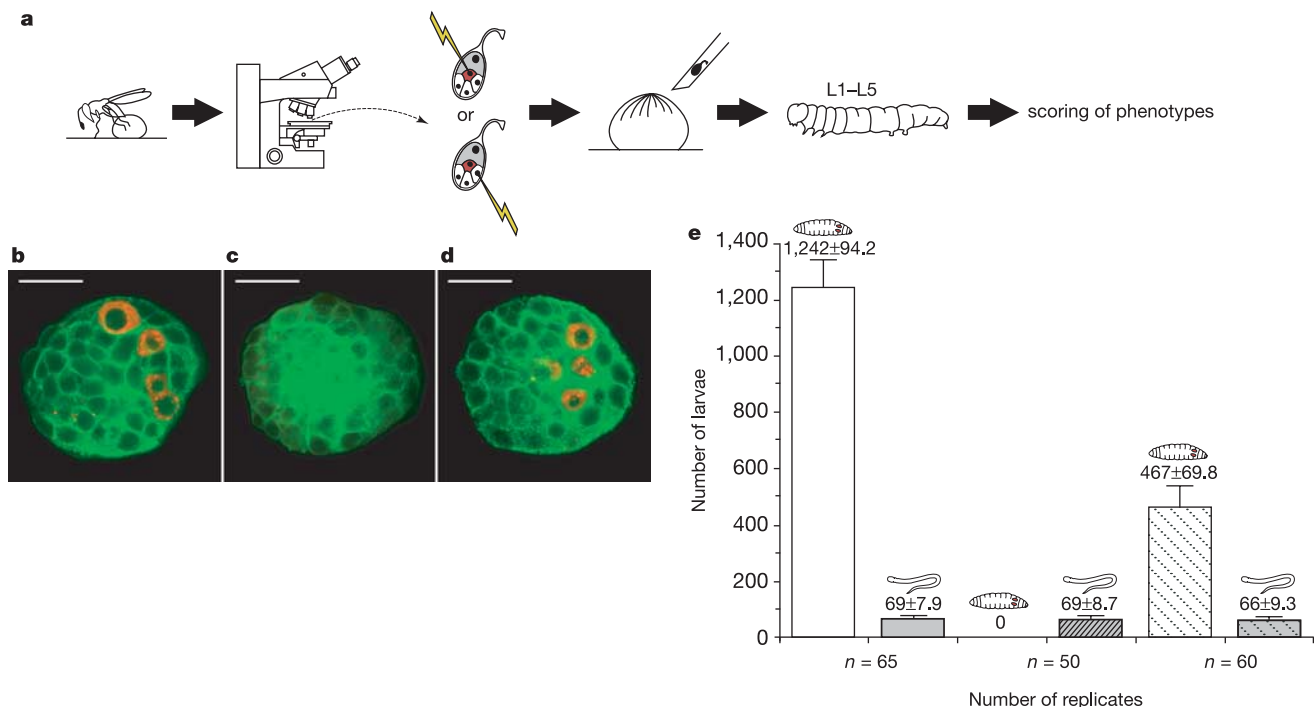


Figure 4 Cell ablation of four-cell stage *Copidosoma* embryo. Red, Vasa protein; green, tubulin. **a**, Experimental design. *Copidosoma* embryos were dissected from the host egg at the four-cell stage. Embryos were either sham-treated, or a small cell (red) was ablated (yellow arrow, upper row), or one of large cells was ablated (yellow arrow, lower row). Embryos were transplanted to the host egg and host larvae were dissected when they reached the fifth larval instar. We counted numbers and examined the morphology of precocious and reproductive larvae to determine the effect of cell

ablation on embryo proliferation and development. **b–d**, Short-term culture of *Copidosoma* embryos *in vitro*. **b**, Sham-treated embryo. **c**, Vasa-positive cell ablated. **d**, Large cell ablated. Scale bars 25 μ m. **e**, Effect of cell ablation on the development of precocious and reproductive larvae. Results are means $\pm$ s.e.m. Solid bars, sham treatment; dark-hatched bars, Vasa-positive cell ablation; light dotted bars, large cell ablation.

When progeny of large-cell-ablated embryos were dissected, we found that both precocious and reproductive larval morphs were produced, although with reduced numbers of reproductive larvae (38%) compared with sham-treated embryos ($t = 6.6$; $P < 0.001$). Cumulatively, these manipulations show that the Vasa-positive cell at the four-cell stage specifies the fate of reproductive larvae and has a major effect on embryo proliferation in *Copidosoma*. The other cells modulate the level of proliferation of reproductive larvae. Surprisingly, the development of precocious larvae was not different between treatments ($P > 0.81$; three pairwise comparisons), indicating that they develop independently of the reproductive lineage and possess a mechanism to compensate for ablated cells.

In contrast with monoembryonic eusocial Hymenoptera, *Copidosoma* castes form clonally from the same zygote in the same host environment. Our data reveal that this unique polyphenism is due to the evolution of a novel developmental process for specification of the sterile and reproductive castes. Instead of environmental cues for the determination of caste sterility, *Copidosoma* relies on a developmental process that distributes germ-cell precursors to reproductive embryos but not to the sterile precocious embryos. This process could rely on a mechanism that uses the differential sorting of Vasa-positive cells to future reproductive embryos; alternatively, differential distribution of germ-cell precursors could be achieved by early downregulation of the germline developmental programme in a subpopulation of Vasa-positive cells in proliferative morulae destined to become precocious embryos.

Our previous work documented many similarities between the development of *Copidosoma* and mammalian embryos, indicating convergent morphological evolution as a result of development in a nutritive host environment⁸. The present results functionally demonstrate that *Copidosoma* combines pre-patterning with regulative abilities in early embryonic development. Our finding parallels recent evidence that mammals might also use pre-patterning in early development^{22,23}. *Copidosoma*, like many invertebrate embryos, segregates determinants that function to specify the germ line. However, early cleavage cells in *Copidosoma* are unusual in that they segregate determinants for cellular proliferation and caste fate rather than determinants for embryo axial polarity. Our data argue for a model in which reproductive embryos represent a mixed lineage of large cells and Vasa-positive cell progeny. The non-cell-autonomous function of Vasa-positive cells is consistent with the model in which these cells serve as signalling centres inducing the proliferation of reproductive embryos. Our data show that species with complex development might have evolved previously unsuspected cellular mechanisms that contribute to developmental plasticity, underscoring a cellular context in the evolution of developmental programmes. □

Methods

Isolation of vasa mRNA, phylogenetic analysis and immunocytochemistry

A *Cfvasa* genomic fragment was isolated by polymerase chain reaction (PCR) with the following degenerate primers: 5'-CAGACGGGTCIGGIAARAC-3' and 5'-TGGAGACG RTCCICRTGDAT-3'. A *Copidosoma* morphogenetic complementary DNA library was screened with a ³²P-labelled PCR fragment to isolate a 2,550-base-pair *vasa* cDNA (GenBank accession number AY611624). The sequences were aligned with a CLUSTAL W algorithm²⁴ using the Gonnet protein matrix, a gap-opening penalty of 10 and a gap-extension penalty of 0.2, using CLUSTAL W v. 1.81 (Linux). An unrooted dendrogram was generated with the maximum-likelihood method²⁵ with 10,000 bootstrap iterations and the computation of clock-like maximum-likelihood branch lengths with TREE-PUZZLE v. 5.1 (Linux). The following Vasa protein sequences were used to create the dendrogram: *Caenorhabditis elegans*: GLH-1 (P34689), GLH-2 (AAB03337), GLH-3 (AAC28388) and GLH-4 (AAC28387); *Hydra magnipapillata*: CnVAS1 (BAB13307) and CnVAS2 (BAB13308); *Ephydatia fluviatilis*: PoVAS1 (BAB13310); *Dugesia dorotocephala*: PIVAS1 (BAB13310); *Drosophila melanogaster*: VASA (P09052); *Bombyx mori*: BmVLG (BAA19572); *Ciona intestinalis*: CiDEAD1A (BAA36711); *Danio rerio*: VLG (CAA72735); *Oncorhynchus mykiss*: OmVASA (BAA88059); *Xenopus laevis*: XLVGI (AAC03114); *Gallus gallus*: CVH (BAB12337); *Mus musculus*: MVH (Q61496); *Rattus norvegicus*: RVLG (Q64060); *Copidosoma floridanum*: CFVAS (AY611624). *In situ* hybridization was

performed with a fragment of the *Cfvasa* cDNA spanning nucleotides 991–1920 as described in ref. 26. Antibody staining, using anti-*Drosophila* Vasa antibody²⁷ (donated by P. Lasko) at a dilution of 1:3 was performed as described in ref. 7.

Laser ablation, embryo culture and embryo transplantation

Copidosoma embryos at the four-cell stage were dissected from the host egg in Ringer's solution²⁸ and transferred to a depression slide. Embryos were sham-treated or individual cells were laser-ablated under a 63 × water-immersion objective on a Zeiss Axioplan II microscope fitted with a Micropoint laser ablation system. After treatment, the embryos were transferred to a bevelled glass microsyringe (60 µm diameter) and transplanted into 0–4-hour-old *T. ni* eggs with a Narashige micromanipulator fitted with an Eppendorf manual air microinjector. Emerged host larvae were reared on an artificial diet as described in ref. 29. Success rates of transplantation were as follows: sham-treated, 27% (241 host larvae developed after transplantation; 65 contained *Copidosoma* embryos); Vasa-positive cell ablated, 20% (251 host larvae developed after transplantation; 50 contained *Copidosoma* embryos); large cell ablated, 20% (299 host larvae developed after transplantation; 60 contained *Copidosoma* embryos). Embryos were cultured *in vitro* as described in ref. 30.

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Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA

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The extreme polymorphism in the human leukocyte antigen (HLA) class I region of the human genome is suggested to provide an advantage in pathogen defence mediated by CD8⁺ T cells^{1–3}. HLA class I molecules present pathogen-derived peptides on the surface of infected cells for recognition by CD8⁺ T cells. However, the relative contributions of HLA-A and -B alleles have not been evaluated. We performed a comprehensive analysis of the class I restricted CD8⁺ T-cell responses against human immunodeficiency virus (HIV-1), immune control of which is dependent upon virus-specific CD8⁺ T-cell activity^{4,5}. In 375 HIV-1-infected study subjects from southern Africa, a significantly greater number of CD8⁺ T-cell responses are HLA-B-restricted, compared to HLA-A (2.5-fold; $P = 0.0033$). Here we show that variation in viral set-point, in absolute CD4 count and, by inference, in rate of disease progression in the cohort, is strongly associated with particular HLA-B but not HLA-A allele expression ($P < 0.0001$ and $P = 0.91$, respectively). Moreover, substantially greater selection pressure is imposed on HIV-1 by HLA-B alleles than by HLA-A (4.4-fold, $P = 0.0003$). These data

indicate that the principal focus of HIV-specific activity is at the HLA-B locus. Furthermore, HLA-B gene frequencies in the population are those likely to be most influenced by HIV disease, consistent with the observation that B alleles evolve more rapidly than A alleles^{6–8}. The dominant involvement of HLA-B in influencing HIV disease outcome is of specific relevance to the direction of HIV research and to vaccine design.

Despite apparently similar roles in pathogen defence, there are well-established differences between the class I loci. HLA-C alleles are expressed at lower levels on the cell surface than HLA-A and HLA-B (ref. 9) and thus it is not unexpected that HLA-C shows the least diversity of the three classical HLA class I loci. However, an unexplained observation is that greater diversity exists at the HLA-B locus than at the HLA-A locus. Overall, there are 563 HLA-B alleles described, compared with 309 HLA-A alleles and 167 HLA-C alleles (<http://www.ebi.ac.uk/imgt/hla/docs/release.html>). It appears that the HLA-B locus is diversifying more rapidly than the HLA-A locus; the significance of which remains unknown.

In order to test the hypothesis that the observed differential diversity at the class I loci reflects functional differences between the HLA-A, -B and -C-restricted CD8⁺ cytotoxic T lymphocyte (CTL) responses, we investigated the contribution of different HLA class I molecules within the CD8⁺ T-cell activity directed against HIV—a pathogen whose control is strongly influenced by CD8⁺ T cells.

We used an empirical approach to determine the contributions of individual HLA class I molecules in anti-HIV host defence in 375 infected, treatment-naïve persons in southern Africa, a region burdened with more HIV infections than any other (<http://www.unaids.org>). This avoids the heavy biases inherent in the use of previously defined optimal epitopes that largely favour particular, well-studied, HLA class I molecules. We employed a panel of 410 overlapping synthetic peptides, spanning the entire expressed HIV genome, and characterized the T-cell responses to these peptides in interferon- γ enzyme-linked immunospot (elispot) assays. Previous studies have validated this approach, and have shown that the responses detected by this method are, with few exceptions, the result of CD8⁺ T-cell activity¹⁰.

This analysis revealed marked differences in the frequency of targeting of individual peptides; 132 peptides being targeted by no subjects, and five peptides targeted by >25% of persons. For many targeted peptides, we noted a strong association with specific class I allele expression: 180 peptides exhibited a strong allele-specific association ($P < 0.05$), of which 111 remained significant ($P < 0.001$) following correction for multiple comparisons (see Supplementary Tables 1 and 2). These included 58 previously defined epitopes (http://www.hiv.lanl.gov/content/immunology/tables/ctl_summary.html). To demonstrate further that the HLA-peptide associations reflect true HLA class I restricted CD8⁺ T-cell responses, we experimentally defined the restriction elements for 12 additional, randomly selected epitopes (Fig. 1, Supplementary Fig. 1 and Supplementary Tables 1 and 2). In each case the restricting allele was precisely that predicted by the statistical association. This analysis therefore affords a stringent approach to define the HLA class I restriction for the large majority of CD8⁺ T-cell responses detected in this population.

We next used this approach to determine the relative contributions of the different class I alleles to immune recognition of HIV-1. The association of HLA-B alleles with peptide-specific responses far exceeded that of HLA-A alleles (67 versus 25/111, $P = 0.0033$, two-tailed unpaired t -test; mean peptide associations per allele, 3.94 versus 1.56) and of HLA-C alleles (19/111, $P = 0.004$; Fig. 2a and b). Of the 30 most highly targeted peptides, 67% were HLA-B-restricted responses (Fig. 2c). Thus, HLA-B alleles contribute significantly more towards the total HIV-specific

CD8⁺ T-cell response than either HLA-A or HLA-C in this population.

We next examined the influence of individual HLA alleles on the level of plasma viraemia, a strong predictor of time to AIDS in B-clade infection¹¹. HLA allele expression and viral load were documented in an extended cohort ($n = 706$) of antiretroviral therapy-naïve, chronically infected Zulu/Xhosa study subjects. Viral load did not vary in the cohort in relation to particular HLA-A allele expression ($P = 0.914$, one-way analysis of variance; Fig. 3a), but varied significantly according to the particular HLA-B allele expressed ($P < 0.0001$), suggesting that an individual's HLA-B type has a profound influence on viral load. In order to identify the particular alleles responsible for differential outcome, as indicated by viral load, we next sought individual alleles for which there were significant differences between the viral loads in subjects who either expressed or did not express the allele. Of 10 significant associations identified (uncorrected, $P < 0.05$), seven were through HLA-B alleles (Fig. 3b). Four of five associations that remained significant after correction for multiple comparisons ($P < 0.001$) were through HLA-B alleles; none was through HLA-A. Thus, the majority of alleles associated with either higher or lower than expected viral loads were HLA-B.

Re-analysis of these HLA associations with high/low viral load indicated that the apparent associations through HLA-A*0205, HLA-Cw*0401 and HLA-Cw*0602 were explained by linkage disequilibrium with HLA-B*5801, HLA-B*8101 and HLA-B*5802, respectively (Fig. 3c, Supplementary Table 3). In contrast, with one exception, the seven HLA-B associations were not altered by linkage

disequilibrium effects. Thus, all six alleles (four, following correction for multiple comparisons) independently associated with either higher or lower than expected viral loads were HLA-B. Together, these data show that HLA-B alleles bear the major burden of HIV-specific CTL activity in chronic HIV infection, and that these are the alleles principally associated with diverse outcomes from HIV infection in this population.

In order to test further the inference that it is particular HLA-B allele expression that principally influences disease outcome, we analysed the absolute CD4 counts in the same cohort ($n = 706$) of chronically infected Zulu/Xhosa study subjects in relation to HLA type. Although there is no recognised steady-state for CD4 count as there is for viral load in chronic infection, CD4 T cell counts are an independent predictor of disease progression in B- and C-clade infection^{11,12}, and a close relationship was also observed between particular HLA-B alleles expressed and disease outcome inferred by CD4 count (Supplementary Fig. 2).

These studies suggest a dominant role for HLA-B alleles in successful or unsuccessful immune containment of HIV infection. To determine whether B alleles exert stronger immune selection pressure on HIV (ref. 13), we examined HIV amino-acid sequence variation in relation to HLA allele expression. Analysis of Nef and Gag, the most immunogenic proteins, demonstrated 25 viral polymorphisms in the Durban cohort associated with HLA-B, 12 with HLA-A and 9 with HLA-C. Eleven of the 46 polymorphisms identified lie within previously defined epitopes, of which ten are presented by the relevant HLA-B allele (data for Nef are shown in Fig. 4a). To investigate further the differential impact of class I loci

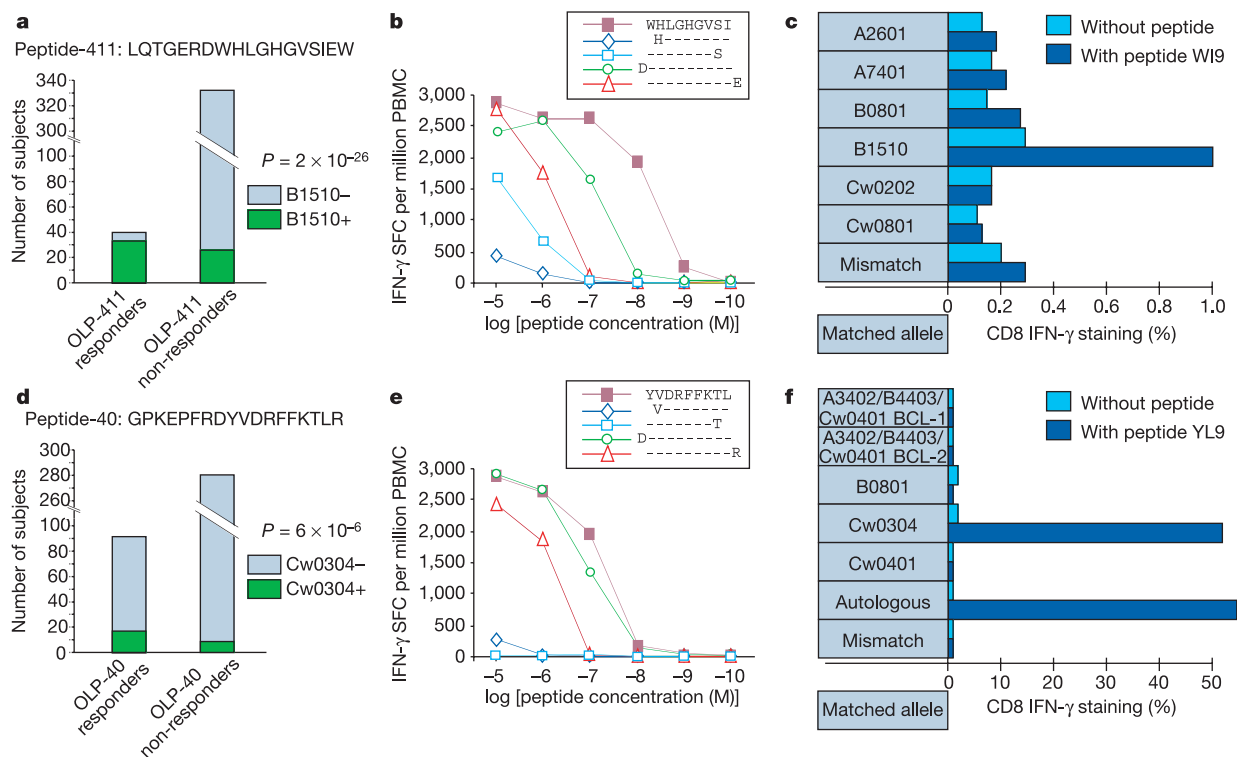


Figure 1 Epitope optimization and formal definition of HLA restriction from HLA-peptide associations. (Total number of subjects, $n = 375$). **a**, Recognition of peptide-411 associated with HLA-B*1510 expression. **b**, WHLGHGVSII (W19) is the optimal epitope. **c**, HLA-B*1510 is the restriction element. Intracellular interferon- γ (IFN- γ) staining assay using PBMC from donor SK-031 (HLA-A*2601/*7401 B*0801/*1510 Cw*0202/*0801) and BCL matched through individual HLA class I molecules as shown. **d**, Recognition of

peptide-40 associated with HLA-Cw*0304 expression. (Total number of subjects, $n = 375$). **e**, YVDRFFKTL (Y19) is the optimal epitope. SFC: spot forming cells; PBMC: peripheral blood mononuclear cells. **f**, HLA-Cw*0304 is the restriction element. Intracellular interferon- γ staining using Y19-specific CTL line from donor 1157-M (HLA-A*3402/*B*0801/*4403 Cw*0304/*0401) and BCL matched through individual HLA class I molecules as shown.

on HIV-1 sequence polymorphism, we analysed a previously reported cohort from Western Australia¹⁴. The HIV-1 Pol sequence polymorphisms in this B-clade infected cohort had shown 12 strong associations with particular HLA class I allele expression, of which 11 were with HLA-B alleles. HLA-C allele associations were not analysed¹⁴. We re-analysed these data to include HLA-C alleles and all the viral proteins. The rates of polymorphism associated with HLA-A, -B and -C were, respectively, 0.0022, 0.0096 and 0.0059 per amino acid (Fig. 4b). Polymorphism was significantly more commonly associated with HLA-B than with HLA-A ($P = 0.0003$). This analysis therefore provides further evidence that HLA-B-restricted CD8⁺ T-cell responses are the most

important in shaping the evolution of HIV.

This study of C-clade infected individuals from the region worst affected by the HIV epidemic shows that most HIV-specific CTL responses are HLA-B-restricted, and that outcome from HIV infection, inferred from viral set-point and CD4 count, is principally associated with particular HLA-B allele expression. Moreover, immune selection pressure on HIV is disproportionately driven by HLA-B alleles. This provides biological evidence to support theoretical data regarding the differential evolution of class I loci, where HLA-B is the most rapidly evolving^{6–8}, and to indicate significant functional differences among individual loci in infectious disease.

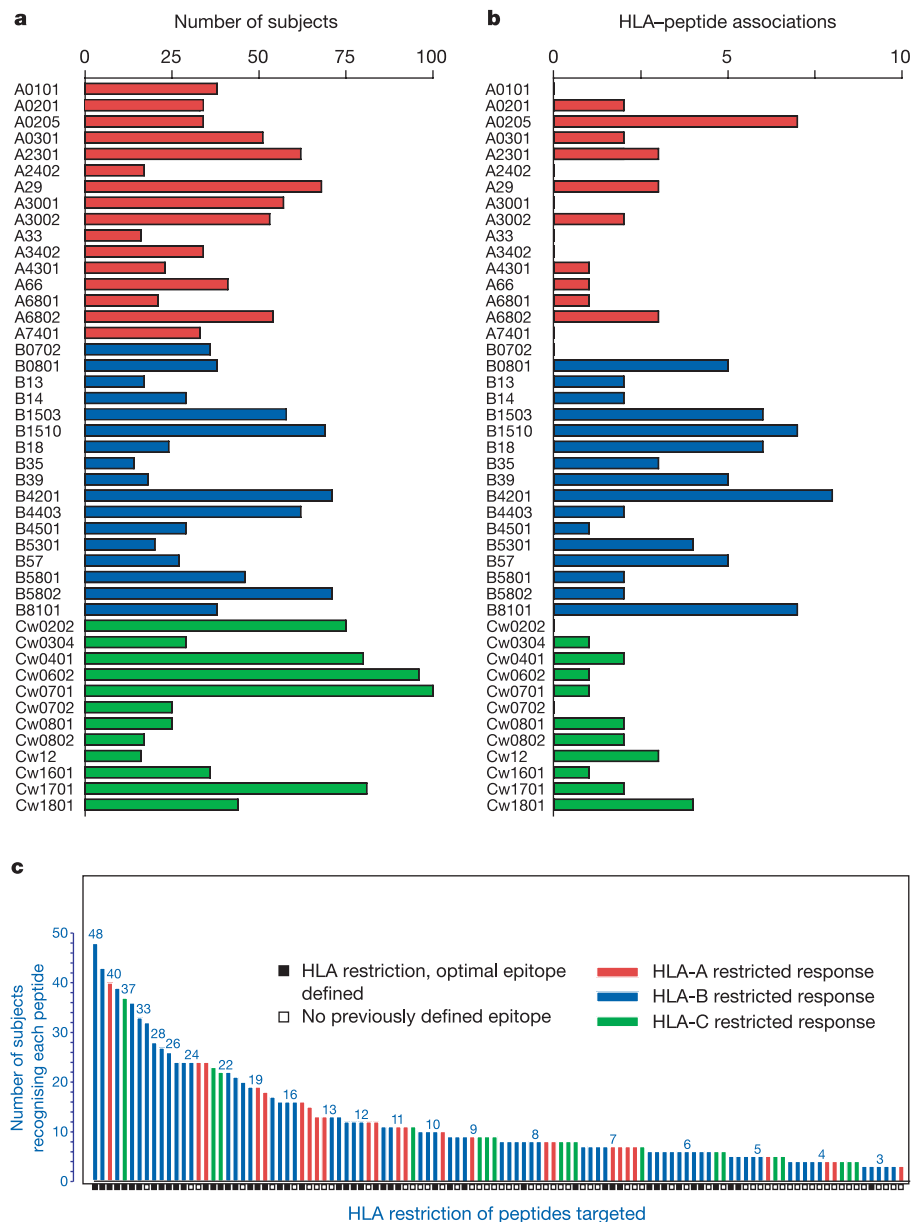


Figure 2 HLA frequencies in study cohort and peptide recognition. **a**, Phenotypic frequencies of HLA class I molecules in the study cohort ($n = 375$) showing alleles present at frequencies of $>3\%$. **b**, Number of peptides recognised in association with individual HLA allele expression. More associations were observed for HLA-B alleles compared to HLA-A alleles ($P = 0.0033$) and for HLA-B compared to HLA-C

($P = 0.0004$). **c**, Number of responders for each peptide for which a peptide–HLA association (in panel **b**) was identified. Filled boxes: previously defined epitope presented by the relevant HLA molecule; open boxes: epitope within the peptide yet to be defined.

These data also support the hypothesis that pathogens controlled in part by the CTL response can adapt and impose selection pressure on the host, particularly on the HLA-B locus. In HIV, both survival and transmission risk depend substantially on viral load^{15,16}, and therefore on the HLA-B alleles expressed. Comparing one generation with the next in this Zulu/Xhosa population, the frequency of deleterious alleles, HLA-B*5802 and B*18, is greater in infected infants (52%, $n = 52$) than in infected antenatal mothers (31%, $n = 511$), while the frequency of protective alleles, B*57 and B*5801, is greater in the infected antenatal mothers (19% versus 6%; $P = 0.0013$, Fig. 5). These data suggest that the frequencies of B*18 and B*5802 will decrease, and those of B*57 and B*5801 will increase rapidly in the population as the epidemic progresses.

These conclusions are supported by previous studies in B-clade infected Caucasians which show that HLA-B alleles are most closely associated with non-progression/low viral load^{17–19} or progression/high viral load^{4,18,20}. Although our study was cross-sectional, and therefore lacks the statistical power afforded to longitudinal studies, the associations between B*57 and B*5801 with low viraemia, and between B*18 and B*5802 with high viraemia, were sufficiently strong to stand out. The dominant effect of HLA-B-restricted CTL

responses on HIV outcome is also supported by work that shows HLA class I homozygous disadvantage in HIV infection⁴. The relative hazards for progression to AIDS or death were 2- to 3-fold higher for HLA-B homozygosity compared with HLA-A homozygosity. Moreover, studies of rare supertype advantage demonstrated an HLA-B effect only²¹.

Although these data indicate a dominant role for HLA-B alleles in HIV infection, the mechanism underlying these observations is unknown. The effect is unlikely to be due to differences in NK cell activation through the HLA-B Bw4 motif²², because the related alleles HLA-B*57, B*5801 and B*5802, associated with diverse outcomes, express the identical Bw4 motif⁵. Moreover, data describing the epistatic interaction between KIR3DS1 and HLA-B indicated that HLA-B*57- and B*27-mediated protection was entirely independent of this interaction²³.

One possible mechanism for the dominant HLA-B allele effect is the greater diversity of peptides bound by HLA-B alleles³. Amino acids binding the B pocket of HLA-A alleles are, without exception, broadly hydrophobic residues (excluding proline, however). In contrast, the B pocket of HLA-B alleles can variously accommodate these residues; but also proline, positively and negatively charged residues, and histidine and glutamine³. The principal source of

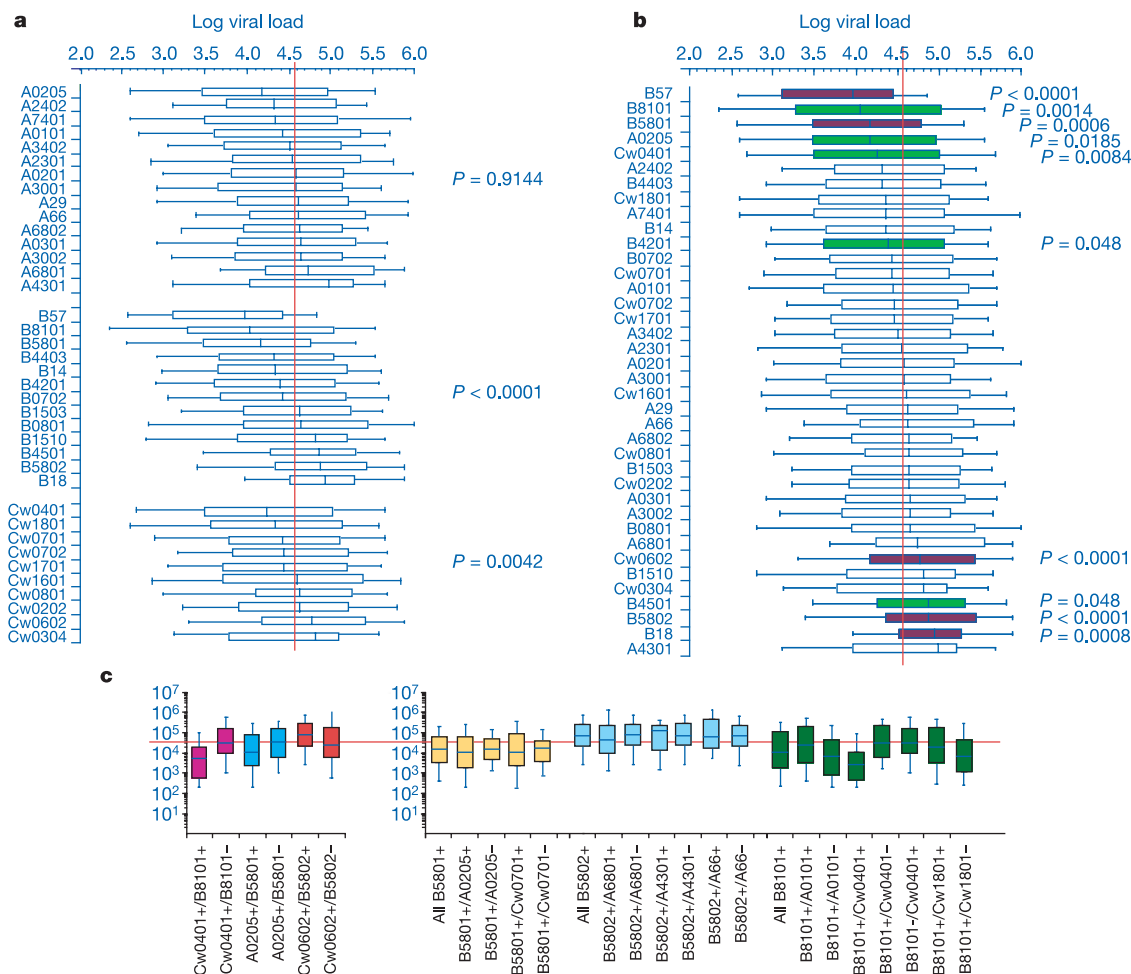


Figure 3 HLA class I molecule expression and viral load in chronically infected Zulu/Xhosa ($n = 706$). **a**, Contribution of class I loci to viral load variation. Viral loads shown as box plots (95th, 75th, 50th, 25th and 5th centiles); vertical red line indicates cohort median viral load. **b**, Association of individual HLA alleles with differential viral loads. Purple shading: $P < 0.001$. Green shading: $0.05 > P > 0.001$. **c**, Effect of linkage

disequilibrium on the associations observed in **b**. Left panel: viral load associations of HLA-Cw*0401, A*0205 and Cw*0602 are lost. Right panel: viral load association of HLA-B*8101 is lost; however, HLA-B*5801, B*5802 (shown), and B*57, B*4201 and B*4501 (not shown) associations are maintained irrespective of linkage disequilibrium effects.

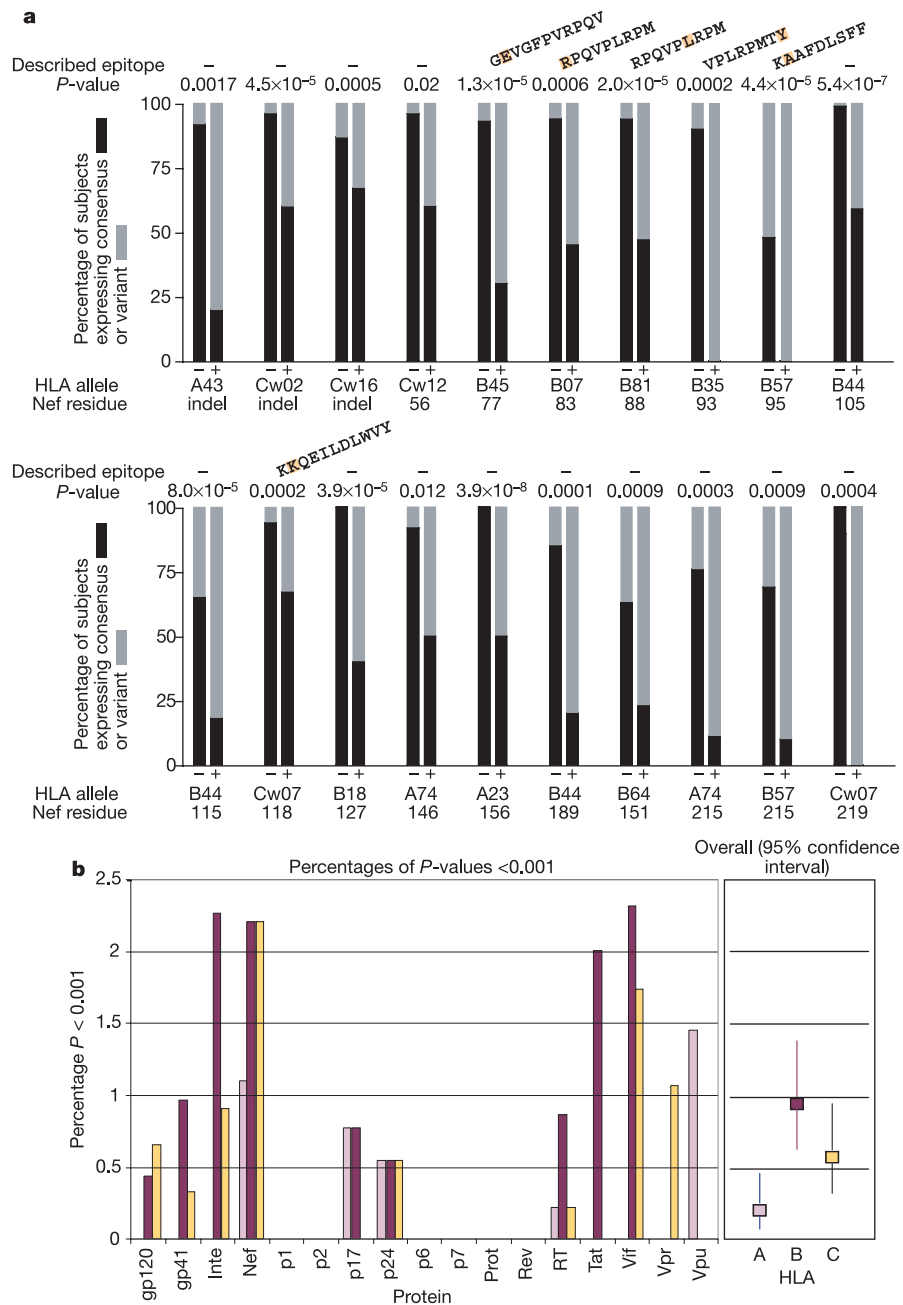


Figure 4 Expression of HLA class I molecules and HIV sequence polymorphism.

a, Analysis of Nef sequences from 123 subjects from Durban, South Africa. Pairs of bars indicate the frequency of individuals whose viral sequence is consensus (black bars) or variant (grey bars) in subjects either expressing (+) or not expressing (–) the relevant allele. Twenty-one associations between expression of particular HLA class I alleles and sequence polymorphisms were identified, as shown. Associations identified within defined epitopes presented by the relevant HLA allele are as shown. Indel, insertion/deletion.

b, Percentages of amino-acid positions at which polymorphism away from population consensus was significantly associated with the HLA-A, -B and -C allelic groups ($P < 0.001$). Numbers of positions considered: 14–263 per protein; a total of 2713 positions across all proteins. Comparisons (McNemar tests): $P = 0.0003$ and $P = 0.12$ for HLA-B versus HLA-A and HLA-C, respectively; $P = 0.04$ for HLA-C versus HLA-A.

HLA-B diversity, however, arises from intralocus recombination events within exon 3, primarily affecting the F, C and D pockets³. The three amino acid differences between HLA-B*5801 and B*5802, for example, would be predicted only to affect the C pocket. We speculate that a variable pathogen such as HIV may adapt more readily to the homogeneous terrain represented by HLA-A alleles, and less so to the functionally diverse HLA-B. This greater functional diversity of HLA-B suggests that, even as the epidemic evolves, the dominant focus of immune-mediated selection pressure

will remain channelled through HLA-B alleles, though not necessarily through the particular alleles predominantly involved today.

These data indicate marked functional differences between HLA-A and -B alleles to the HIV-specific CD8⁺ T-cell response. Differences in viral load and absolute CD4 count, and, by inference, in HIV disease outcome, are principally related to HLA-B allele expression, as is HLA-mediated immune selection pressure. This work does not prove a co-evolutionary process between HIV and

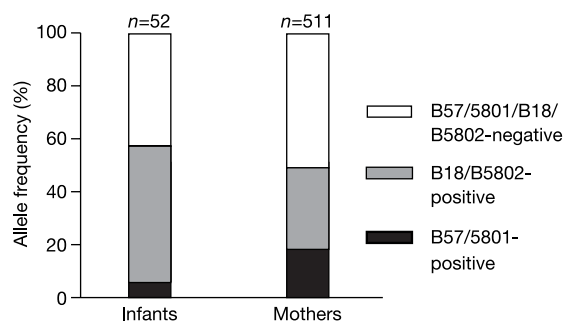


Figure 5 Altered frequencies of alleles associated with high or low viral set-point in infected infants and mothers. Frequencies of protective alleles (B*57 and B*5801) and susceptibility alleles (B*18 and B*5802) differed significantly in the two groups ($P = 0.0013$, Fisher's exact test).

HLA—which would require the availability of samples derived from the two co-evolving genetic entities obtained over great time periods. However, it provides a snapshot in time that is highly indicative of these dynamics in populations where HIV disease is showing high morbidity and mortality rates. The previous observation that HLA-B is evolving more rapidly than the HLA-A or -C loci suggests that, for pathogens in addition to HIV whose control depends upon the CD8⁺ T-cell response, similar effects may be observed. The immunogenetic analyses of human diseases undertaken to date largely support this hypothesis^{24–26}, and suggest a dominant involvement of HLA-B alleles in the control of human pathogens. □

Methods

Study subjects

Characterization of HIV-specific T-cell responses was undertaken in study subjects from southern Africa. Of the 375 subjects studied, 314 were from Durban, South Africa, the remainder being from Zimbabwe, Angola, Malawi, Zambia and Botswana. In 219 cases, asymptomatic subjects were tested for HIV infection during pregnancy following voluntary counselling, and were studied subsequent to diagnosis; 156 subjects were recruited from outpatient follow-up clinics. This latter group were mostly diagnosed following clinical presentation suggestive of HIV infection. The subjects for whom analysis of HLA type and viral load was performed ($n = 706$) were all antiretroviral therapy naive, from Durban, South Africa. This group comprised 494 asymptomatic subjects recruited from antenatal clinics as described above and 212 subjects from out-patient follow-up clinics. Fifty-three of these 706 enrolled subjects (7.5%) were males. Overall, the median viral load in this cohort was 37,500 and the median absolute CD4 count was 387. Viral load measurement was undertaken using the Roche Amplicor version 1.5 assay.

Elispot, HLA restriction assays

Four hundred and ten 18-mer peptides, spanning the expressed entire HIV genome, were synthesized on the basis of the consensus of available C-clade sequences in 2001. These peptides were used in a matrix system of 11–12 peptides per pool to screen study subjects for HIV-specific T cell responses by interferon- γ elispot assay, as previously described¹⁰. Confirmation of recognised individual 18-mer peptides within a peptide pool was undertaken in a separate elispot assay. Formal definition of the HLA restriction of the responses was carried out either by intracellular cytokine staining or by elispot assay using PBMC or CTL lines as effectors.

HLA typing

For the subjects studied in the Durban cohort, genomic DNA samples were initially typed to an oligo-allelic level using Dynal RELITM reverse Sequence Specific Oligonucleotide (SSO) kits for the HLA-A, -B and -C loci (Dynal Biotech). Refining the genotype to the allele level was performed using the Dynal Biotech sequence-specific priming kits in conjunction with the previous SSO type. Where alleles were still not defined to the allele level, bespoke sequence-specific priming primer mixes were utilised. All HLA class I alleles in the IMGT allele release 2.4.0 were considered in the typing²⁷. HLA class I typing for the Perth cohort was performed by direct sequencing methods¹⁴.

Viral sequencing

Sequencing of HIV proviral DNA from patients in the Durban cohort was undertaken as previously described²⁸. Sequencing of proviral DNA and viral RNA from patients in the Perth cohort was undertaken as described¹⁴.

Statistics

See Supplementary Information.

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Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to P.J.R.G. (philip.goulder@ndm.ox.ac.uk). GenBank accession numbers and Full Gag and Nef sequence alignments for the sequences from Durban, South Africa, included in this study are available at (http://www.hiv.lanl.gov/content/immunology/hlatem/south_africa_sequences/) and other accession numbers are available in the Supplementary Information.

***Fbxw7/Cdc4* is a p53-dependent, haploinsufficient tumour suppressor gene**

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The *FBXW7/hCDC4* gene encodes a ubiquitin ligase implicated in the control of chromosome stability¹. Here we identify the mouse *Fbxw7* gene as a p53-dependent tumour suppressor gene by using a mammalian genetic screen for p53-dependent genes involved in tumorigenesis. Radiation-induced lymphomas from p53^{+/-} mice, but not those from p53^{-/-} mice, show frequent loss of heterozygosity and a 10% mutation rate of the *Fbxw7* gene. *Fbxw7*^{+/-} mice have greater susceptibility to radiation-induced tumorigenesis, but most tumours retain and express the wild-type allele, indicating that *Fbxw7* is a haploinsufficient tumour suppressor gene. Loss of *Fbxw7* alters the spectrum of tumours that develop in p53 deficient mice to include a range of tumours in epithelial tissues such as the lung, liver and ovary. Mouse embryo fibroblasts from *Fbxw7*-deficient mice, or wild-type mouse cells expressing *Fbxw7* small interfering RNA, have higher levels of Aurora-A kinase, c-Jun and Notch4, but not of cyclin E. We propose that p53-dependent loss of *Fbxw7* leads to genetic instability by mechanisms that might involve the activation of Aurora-A, providing a rationale for the early occurrence of these mutations in human cancers.

Mice carrying inactivated alleles of the p53 gene develop lymphomas and sarcomas, although epithelial tumours are relatively uncommon². Heterozygous mice with one functional p53 allele develop tumours after a considerably longer latency period than their homozygous null counterparts^{2,3}, possibly because of the requirement for other genetic events for tumour development. We have compared the spectrum of genetic alterations in p53-null tumours that had developed in p53 heterozygous animals with similar tumours from p53-null mice. If loss of the wild-type p53 allele in heterozygous mice is the first somatic genetic event, we would expect the overall pattern of genetic changes to be very similar to that of tumours from p53-null mice. However, if additional events are required for the growth or survival of somatic cells that suffer a second hit in the p53 gene, we would expect to find certain genetic changes exclusively in tumours from the p53 heterozygous mice.

We generated F₁ hybrid and backcross mice from two different species (*Mus spretus* and *Mus musculus*), one of which (the 129/Sv strain of *M. musculus*) has no functional p53 gene, and compared the spectrum of genetic alterations in tumours from p53^{+/-} F₁ hybrid and backcross mice, with that from the F₁ backcross p53^{-/-} mice. Over 100 microsatellite markers were used to detect genome-wide loss of heterozygosity (LOH; see Supplementary Fig. 1a,b; Fig. 1a). Chromosome 3 showed LOH in almost all tumours from the p53^{+/-} F₁ hybrid mice and in about 50% of the p53^{+/-} F₁ backcross mice, but only background levels in tumours from the p53^{-/-} mice. Chromosomes 16 and 18 also showed p53-dependent

LOH ($P < 0.05$ in all cases), whereas no differences were seen in LOH frequency on chromosomes 12 and 19 in tumours from p53^{+/-} and p53^{-/-} mice (Fig. 1a). Sub-chromosomal losses were detected on chromosome 3 that implicated the interval between markers *D3Mit139* and *D3MA38* (Fig. 1b), which contains several candidate genes implicated in tumorigenesis including *Mts1* (ref. 4), *TrkA* (ref. 5) and *Fbxw7/Cdc4* (refs 1, 6–9). Sequencing of the complete coding region of *Mts1* from 20 tumours that exhibited LOH did not reveal the presence of any inactivating mutations in the remaining allele. Analysis of 85 additional radiation-induced

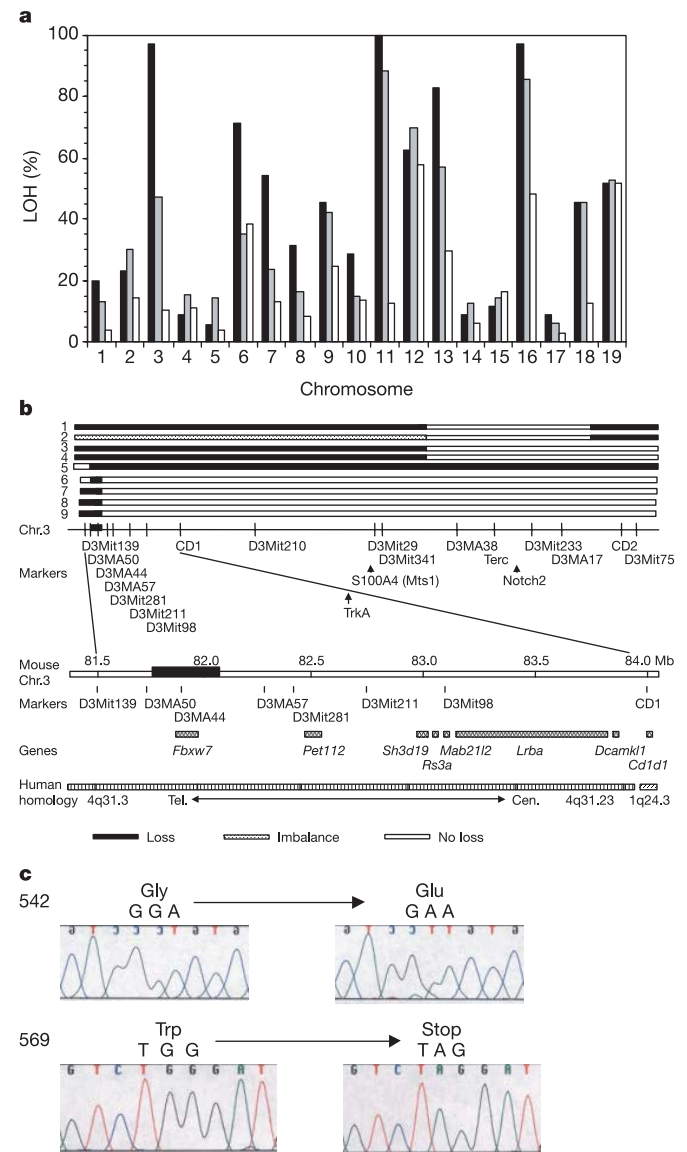


Figure 1 LOH and mutation analysis of radiation-induced lymphoma. **a**, Frequency of allelic deletion or imbalance on each mouse chromosome: black bars, F₁ p53^{+/-} ($n = 35$); hatched bars, F₁ backcross p53^{+/-} ($n = 49$); F₁ backcross p53^{-/-} ($n = 61$). **b**, Detailed sub-localization of deletions on chromosome 3 (chr. 3). Physical mapping of markers and genes, and human homology, is based on the Celera database. Tumour numbers 1–5 are from (*M. spretus* × 129/Sv) hybrids, and tumour numbers 6–9 are from (C57BL/6J × 129/Sv) hybrids. Black bar on chromosome shows common deletion region. Tumour 6 showed clear LOH with the marker *D3MA44* within the *Fbxw7* gene but not at *D3MA50* and *D3MA57*, whereas tumours 7–9 had LOH at *D3MA44* but not at *D3MA57*. Mb, megabases. **c**, Sequence traces showing the mutations of *Fbxw7*. Codon positions in *Fbxw7* α are shown at the left.

lymphomas from (C57BL/6J $\times$ 129/Sv) F₁ hybrid mice (40 p53^{+/-} and 45 p53^{-/-}) defined the distal LOH breakpoint on chromosome 3 between markers *D3MA44* and *D3MA57* (Fig. 1b). One lymphoma showed clear LOH only with the marker *D3MA44*, which lies within the *Fbxw7* gene (also known as *CDC4*, *SCF^{Fbw7}*, *AGO*, *SEL10*)⁶⁻¹¹, encoding an F-box protein that controls the level of several target proteins implicated in oncogenesis^{6,7,10,11-13}. No other expressed sequence tags or transcripts were located within the minimal region, thus identifying *Fbxw7* as the only viable candidate.

The mouse *Fbxw7* coding region from *M. musculus* and *M. spretus* was sequenced and shown to have three splice forms (denoted α , β and γ , respectively, Supplementary Fig. 2a, b), which is compatible with the analysis of human FBXW7/*hCDC4* (ref. 8). The complete coding sequence was determined from 20 tumours from p53^{+/-} mice and a further 20 tumours from p53^{-/-} mice. Although no mutations were found in tumours from the p53^{-/-} mice (which also showed no LOH), two p53^{+/-} mouse tumours had point mutations within one of the WD40 domains generating a truncating stop codon or a missense mutation (Fig. 1c). Similar mutations have been reported in human colon cancers¹. RNA was available from all lymphomas that showed LOH at the *Fbxw7* locus, and in all cases the remaining *Fbxw7* allele was expressed (data not shown), indicating that no gene silencing had occurred.

Because *Fbxw7* LOH and mutations were detected only in

tumours from p53^{+/-} mice and not from p53^{-/-} mice, we further explored the relationship between these two genes. A 10-kilobase promoter region of *Fbxw7* α , β and γ forms was searched for p53 DNA-response elements with the p53MH algorithm¹⁴. We identified nine putative p53 DNA-responsive elements in the *Fbxw7* α promoter region, four in *Fbxw7* β and five in *Fbxw7* γ (see Supplementary Table 1). To test whether activation of p53 by radiation might have an effect on the expression of *Fbxw7*, mouse embryonic fibroblasts (MEFs) from p53 wild-type, heterozygous and null mice were subjected to 4 Gy of γ -radiation and *Fbxw7* expression was assessed by quantitative Taqman analysis. The results showed a consistent increase in the level of *Fbxw7* RNA that was dependent on p53 (Fig. 2a). Taqman analysis was performed at least three times, and a statistically significant increase was found 2.5–7.5 h after exposure to radiation. Figure 2a also shows that the basal levels of *Fbxw7* expression were lower in p53-null than in wild-type MEFs, in agreement with the interpretation that *Fbxw7* is under positive regulation by p53. These results are compatible with previous data on the human FBXW7/*hCDC4* gene showing that it is a direct transcriptional target of p53 (ref. 15).

We designed small interfering RNA (siRNA) against *Fbxw7* and generated stable transfectants in MEFs of different p53 status. The siRNA downregulated *Fbxw7* RNA levels by 75%, as assessed by quantitative TaqMan assays (Fig. 2b). Whereas MEFs from p53

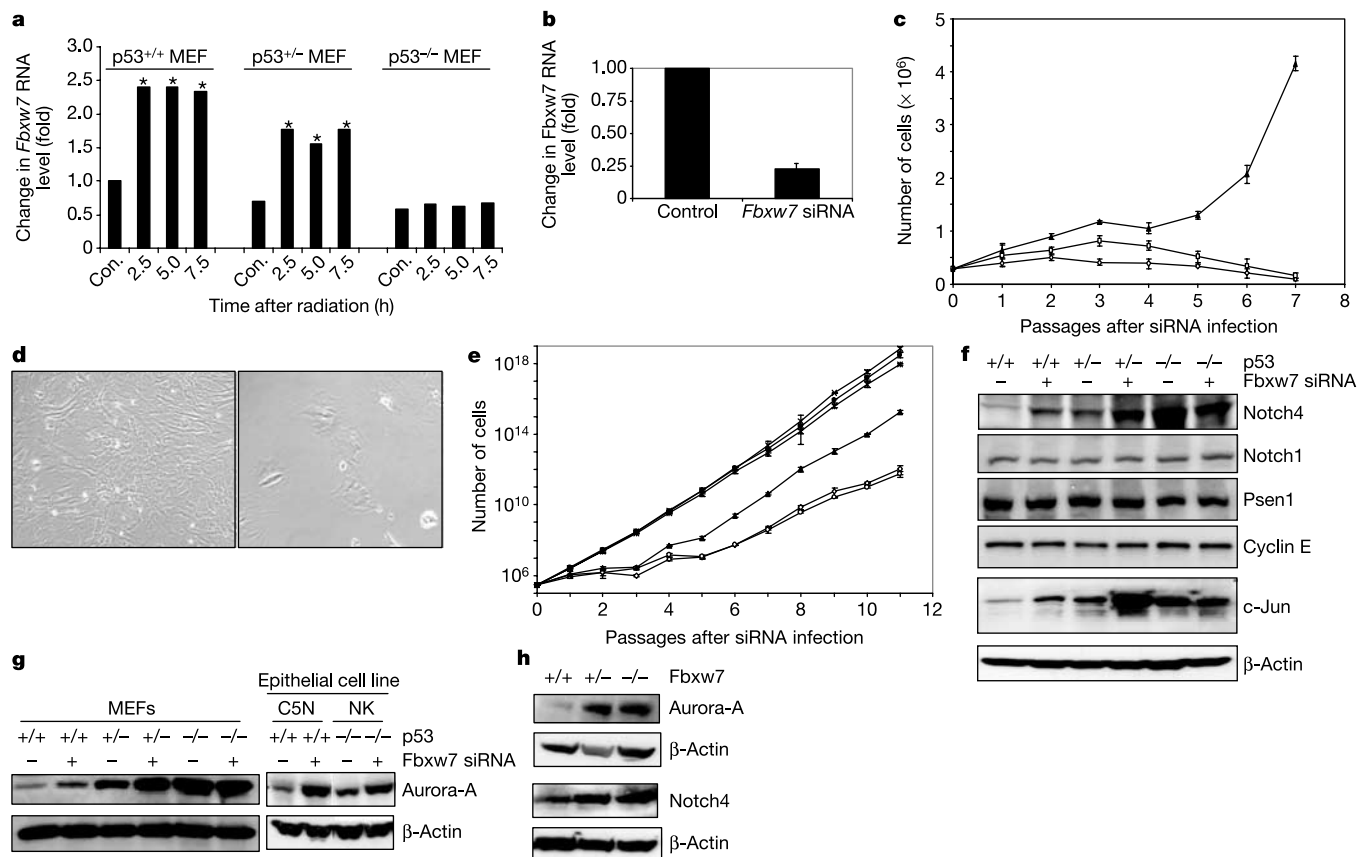


Figure 2 Relationship between *Fbxw7* and p53. **a**, *Fbxw7* mRNA expression in MEFs after irradiation (asterisk indicates $P < 0.05$). **b**, *Fbxw7* RNA downregulation by siRNA. **c**, siRNA against *Fbxw7* caused a delay in replicative senescence. Growth was prolonged by *Fbxw7* siRNA, but the MEFs were not immortalized. Diamonds, wild-type MEFs; squares, wild-type MEFs with *Fbxw7* siRNA control; triangles, wild-type MEFs with *Fbxw7* siRNA. **d**, MEFs from p53 wild-type mice (right) and with *Fbxw7* siRNA (left). **e**, Downregulation of *Fbxw7* in p53^{+/+} MEFs, but not in p53^{-/-} MEFs, induced a growth advantage. Diamonds, p53^{+/+} MEFs; open circles, p53^{+/+} MEFs with *Fbxw7* siRNA control; triangles, p53^{+/+} MEFs with *Fbxw7* siRNA; crosses, p53^{-/-} MEFs; stars, p53^{-/-} MEFs

with *Fbxw7* siRNA control; filled circles, p53^{-/-} MEFs with *Fbxw7* siRNA. **f**, Western blot showing *Fbxw7* target protein levels in MEFs. Notch4 and c-Jun levels were upregulated when *Fbxw7* was downregulated by siRNA in p53^{+/+} and p53^{+/-} MEFs. No changes were found in Notch1, Presenilin 1 (Psen1) and cyclin E. **g**, Western blot showing increased Aurora-A levels when *Fbxw7* is downregulated by siRNA in p53^{+/+} or p53^{+/-} MEFs or keratinocytes. **h**, Aurora-A levels are upregulated in embryonic day 9.5 *Fbxw7*^{+/+} and *Fbxw7*^{-/-} MEFs. Controls were empty vector or constructs expressing other siRNA unrelated to *Fbxw7*. Error bars indicate standard deviation for three independent assays.

wild-type mice entered early senescence, cells expressing the *Fbxw7* siRNA maintained a healthy appearance and, after a slight lag phase, grew progressively (Fig. 2c, d), although they were not immortalized and subsequently senesced (data not shown). Downregulation of *Fbxw7* in p53 heterozygous MEFs also induced a clear growth advantage compared with controls (Fig. 2e), whereas p53-null MEFs showed similar growth properties independently of the presence of *Fbxw7* siRNA (Fig. 2e).

We investigated the protein levels of several known *Fbxw7* targets^{6,7,10–13} in p53 wild-type and deficient MEFs, with and without *Fbxw7* siRNA. Notch4 levels were clearly increased when *Fbxw7* was downregulated in the presence of one or two copies of the wild-type p53 gene, but not cyclin E, Notch1 and Presenilin1 (Fig. 2f). These data are in agreement with observations that *Fbxw7* target proteins are cell- and context-dependent^{16,17}. We conclude that Notch4 levels are regulated by *Fbxw7*, and that deregulation of this or another unknown pathway might be important in the growth of MEFs *in vitro*, and possibly in lymphomagenesis¹⁸.

Loss of FBXW7/hCDC4 has been implicated in the control of genetic instability¹. FACS analysis of MEFs from wild-type mice before and after treatment with *Fbxw7* siRNA showed an increase in the percentage of aneuploid cells, indicating increased genomic instability (see Supplementary Fig. 3). This effect is not due to a loss of p53 in these cells, because we have shown that the wild-type p53 gene is still present and expressed at normal levels in the presence of *Fbxw7* siRNA (see Supplementary Fig. 4). Because the results shown in Fig. 2f indicated that cyclin E deregulation might not be responsible for increased genetic instability in this system, we investigated the possibility that the mitotic control gene *Aurora-A* kinase (also known as *STK15* in human and *Stk6* in mouse)^{19–21} might be under the control of *Fbxw7*. Figure 2g shows that in cells

from wild-type or p53^{+/–} mice, treatment with *Fbxw7* siRNA leads to a clear stimulation of Aurora-A protein levels. Other studies with different MEFs and with p53 wild-type or null keratinocytes showed that the effect of *Fbxw7* downregulation on Aurora-A does not require the presence of p53 (Fig. 2g; data not shown). As an additional control, we tested the levels of c-Jun²² in the same cells used for analysis of Aurora-A expression, and confirmed that knockdown of *Fbxw7* with siRNA increases levels of this target protein (Fig. 2f). *Fbxw7*^{+/–} and *Fbxw7*^{–/–} cells from cells of embryonic day 9.5 embryos showed a clear increase in levels of Aurora-A and Notch4 (Fig. 2h), and in fact the *Fbxw7*^{+/–} cells expressed both proteins to almost the same degree as the null cells. These data are in agreement with results indicating that *Fbxw7* has the properties of a haploinsufficient tumour suppressor gene. Additional studies will be required to determine whether the effects of *Fbxw7* downregulation on Aurora-A levels are direct or indirect, and to identify additional *Fbxw7* substrates in epithelial cells.

We exposed wild-type, *Fbxw7*^{+/–}, p53^{+/–}, *Fbxw7*^{+/–}p53^{+/–}, p53^{–/–} and *Fbxw7*^{+/–}p53^{–/–} mice to a single dose of 4-Gy γ -irradiation at 5 weeks of age to determine whether loss of *Fbxw7* accelerates tumorigenesis *in vivo*. Within 75 weeks, no spontaneous tumours were detected in more than 65 unirradiated *Fbxw7*^{+/–} mice, whereas more than 30% of irradiated mice developed tumours (Fig. 3a). Loss of one allele of *Fbxw7* also accelerated tumorigenesis in irradiated p53^{+/–} mice ($P = 0.028$ for tumorigenesis in p53^{+/–} versus p53^{+/–}*Fbxw7*^{+/–} mice), but no acceleration was seen in p53^{–/–} mice ($P = 0.9$ for tumorigenesis in p53^{–/–} versus p53^{–/–}*Fbxw7*^{+/–} mice). Tumours from the *Fbxw7*^{+/–}p53^{+/–} double heterozygous mice showed loss of the remaining *Fbxw7* allele in only two of ten tumours investigated (Fig. 3b), and expression of *Fbxw7* was detected at the RNA level in six tumours that had retained the wild-type allele (Fig. 3c). We conclude that *Fbxw7* has many of the properties of a haploinsufficient tumour suppressor gene^{23–24}, in that loss of one copy confers predisposition without requiring complete inactivation of the remaining allele.

Irradiated p53^{+/–} mice develop primarily lymphomas, and more rarely fibrosarcomas, but almost never succumb to epithelial tumours. The double heterozygous p53^{+/–}*Fbxw7*^{+/–} mice developed epithelial lung and liver tumours and a range of lesions in the ovary (Table 1, Fig. 4). Some epithelial tumours were also found in the irradiated *Fbxw7*^{+/–} mice, although after a longer latency period (50 weeks). Other mouse models of cancer that result in genetic instability have also given rise to epithelial tumours. Breeding of *Terc*^{–/–} mice, which lack the RNA component of the enzyme telomerase, through multiple generations leads to telomere shortening and widespread genetic instability. On a p53^{+/–} background, a range of tumours developed that are not normally seen in p53-deficient animals²⁵. Epithelial tumours have also been observed in mice with a germline mutation in the p53 gene, which also causes genetic instability²⁶. Epithelial cell transformation may require a minimum threshold level of genetic instability that is not normally attainable by loss of p53 function. Development of epithelial tumours in irradiated *Fbxw7*-deficient mice suggests that *Fbxw7* might be particularly important in the protection of epithelial tissues from the consequences of DNA damage leading to genetic

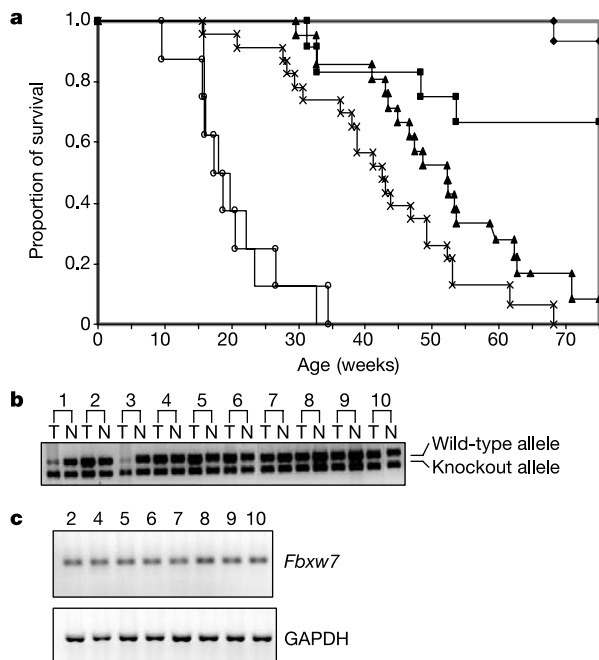


Figure 3 Radiation-induced tumorigenesis in p53-deficient and *Fbxw7*-deficient mice. **a**, Tumour development in p53-deficient and *Fbxw7*-deficient mice: wild-type (diamonds; $n = 15$), *Fbxw7*^{+/–} (filled squares; $n = 12$), p53^{+/–} (filled triangles; $n = 21$), p53^{+/–}*Fbxw7*^{+/–} (crosses; $n = 23$), p53^{–/–} (open circles; $n = 13$) and p53^{–/–}*Fbxw7*^{+/–} (plain line; $n = 16$). **b**, Most tumours from *Fbxw7*^{+/–} mice do not show loss of the wild-type allele of *Fbxw7*: T, tumour DNA; N, normal tail DNA. Tumours 1 and 3 show loss of the *Cdc4* wild-type allele, but retention is seen in most cases. **c**, Continued expression of *Fbxw7* RNA (by RT-PCR) in tumours from *Fbxw7* heterozygous mouse tumours that did not show LOH.

Table 1 Tumour types in p53^{+/–}*Fbxw7*^{+/–} mice ($n = 23$)

Tumour type	Frequency (%)
Thymic lymphoma	43.5
Ovary tumour	17.4
Lung tumour	13.0
Liver tumour	13.0
Fibrosarcoma	13.0
Pancreas tumour	4.3
Osteosarcoma	4.3

instability. An important difference between this model and those already published is the nature of the genetic instability that leads to tumour formation. Telomere shortening leads primarily to bridge–fusion–breakage cycles and translocations²⁵, whereas loss of *Fbxw7* may induce aneuploidy¹ at least in part through the induction of Aurora-A, which is a known mediator of these effects^{19–20}. The recent documentation of cancer-prone families with germline mutations in the mitotic checkpoint gene *BUB1B* supports a causal link between aneuploidy and tumour susceptibility²⁷. This mechanism might explain the development of genetic instability and epithelial tumours in mice in spite of the presence of extremely long telomeres.

Our data suggest a model (Supplementary Fig. 5) in which p53 itself is unlikely to be the primary genetic ‘target’ for deletions in tumours that develop in irradiated p53^{+/–} mice, possibly because of induction of a p53-independent cell death mechanism²⁸. Because all tumours from the p53^{+/–} animals ultimately lose the single copy of the functional p53 gene, the simplest interpretation of the data is that loss of the wild-type allele must be preceded by a loss of genes such as *Fbxw7* that mediate p53 responses to DNA damage. This

interpretation is supported by *in vitro* and *in vivo* data (Figs 2e and 3a) showing that loss of *Fbxw7* does not provide any selective advantage in the cells or mice with no p53 function. However, other possible explanations have not been excluded: developmental adaptation to p53 nullizygosity might result in different selection pressures in tumours from p53^{+/–} versus p53^{–/–} mice, such that *Fbxw7* inactivation might occur after p53 loss. Studies on the relative timing of these genetic events in early-stage lymphomas from p53^{+/–} mice would be required to resolve this question. □

Methods

Mice and tumour induction

F₁ hybrid mice were generated by crossing female p53^{–/–} 129/Sv (ref. 2) with male p53^{+/+} *M. spretus* or C57BL/6J, and F₁ backcross mice obtained by mating the female F₁ hybrids with male p53^{+/–} 129/Sv mice. *M. spretus* mice were from S. Brown (MRC, Harwell, UK). Mice were bred and treated under UK Home Office regulations or according to LARC regulations in the USA. The 5-week-old 129/Sv, F₁ and F₁ backcross mice of both sexes were exposed to a single dose of 4 Gy whole-body irradiation from a cobalt-60 source at a dose rate of 0.7 Gy min^{–1} and observed daily until they were moribund, then killed and autopsied.

p53 and *Fbxw7* single- or double-heterozygous knockout mice were generated by crossing *Fbxw7*^{+/–} (ref. 16) with p53^{+/–} mice. To generate all combinations of genotypes, we crossed male p53^{+/–} *Fbxw7*^{+/–} knockout mice with female p53^{+/–} *Fbxw7*^{+/–} knockout mice. All littermates were irradiated as described above.

LOH and mutation analysis of *Fbxw7*

All tumour DNA samples were examined together with normal DNA from the same mice, to take account of variation in some alleles of the outbred *M. spretus* mice used in these studies by polymerase chain reaction (PCR) (for details see Supplementary Methods).

Mutation analysis of the *Fbxw7* gene was performed on 20 F₁ p53^{+/–} and 20 F₁ backcross p53^{+/–} thymic lymphomas by sequencing. Each exon of the *Fbxw7* gene was amplified from tumour DNA by PCR (for primers see Supplementary Table 3). The PCR products were purified and sequenced directly with cycle sequencing (Amplifycycle sequencing kit; Perkin-Elmer).

Reverse transcription (RT) reactions for RNAs from all F₁ thymic lymphomas were performed with the ThermoScript RT–PCR system (Gibco-BRL) and performed in accordance with the manufacturer’s instructions. The primers for PCR and sequencing are shown in Supplementary Table 4.

Detection of *Fbxw7* mRNA expression by Taqman analysis

p53 wild-type, heterozygous and null MEFs were subjected to 4 Gy of ionizing radiation and were collected at different time points. Total RNA at each time point was used to assess *Fbxw7* expression by Taqman analysis. The primers for *Fbxw7* were 5′-CACAGGCCTTCAA GAGTGGC-3′ (forward) and 5′-TTGCATCATATGCTTCACTTGTGT-3′ (reverse). The probe for *Fbxw7* was 5′-(6-FAM)-AATGTTTCAGAGCTGGAGCGGACCAG-(TAMRA)-3′, where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine. The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5′-TGACCACTCAACTGCTTAG-3′ (forward) and 5′-GGATGCAGGGATGATGTTC-3′ (reverse). The probe for GAPDH was 5′-(6-FAM)-CAGAAGACTGTGGATGGCCCTC-(TAMRA)-3′. *Fbxw7* expression was measured with the ABI PRISM 7700 sequence detection system (Applied Biosystem). PCR reactions contained cDNA (50 ng), 1 × Taqman universal PCR master mix, forward and reverse primers (900 nM) and 200 nM FAM-labelled probe. PCR reactions were performed in triplicate for each sample, and experiments were repeated a minimum of three times. C_t values (the PCR cycle number at which the amplification plot crosses the threshold) were normalized against GAPDH RNA. The fold increase of expression at different time points was calculated by 2^{T–C}, where T is normalized C_t at this time, and C is normalized wild-type control C_t.

siRNA transfection and cell growth assays

MEFs were prepared from mice at embryonic day 13.5 (from p53^{+/–} intercrossed parents) or embryonic day 9.5 (from *Fbxw7*^{+/–} intercrossed parents). All experiments were performed with MEFs prepared from at least two different litters. The MEFs were genotyped by PCR. C5N (p53^{+/+}) and NK (p53^{–/–}) are two well-characterized keratinocyte cell lines²⁹.

C5N cells, NK cells and MEFs were infected with high-titre retroviral stocks produced by transient transfection of 293T ecotropic Phoenix cells. After infection with the pSuper retrovirus (OligoEngine) allowing the expression of siRNA molecules³⁰, cells were selected with 1–2 µg ml^{–1} puromycin in the culture medium. The oligonucleotide for *Fbxw7* siRNA was 5′-CACAAAGCTGGTGTGTGCA-3′, which targets a region in exon 10 (the coding region 1965–1983, GenBank accession number NM_080428). The control of the experiment was empty pSuper vector and other siRNA unrelated to *Fbxw7*: 5′-GTTCCGACTGAACAGACGA-3′.

For MEF proliferation assays, cells were seeded into three 100-mm tissue culture plates at 3 × 10⁵ cells per plate in DMEM (high glucose) medium supplemented with 10% fetal bovine serum and penicillin–streptomycin. Cell numbers were determined every 3 days in a Neubauer chamber. Cumulative cell numbers were calculated at each passage. Experiments were repeated at least three times.

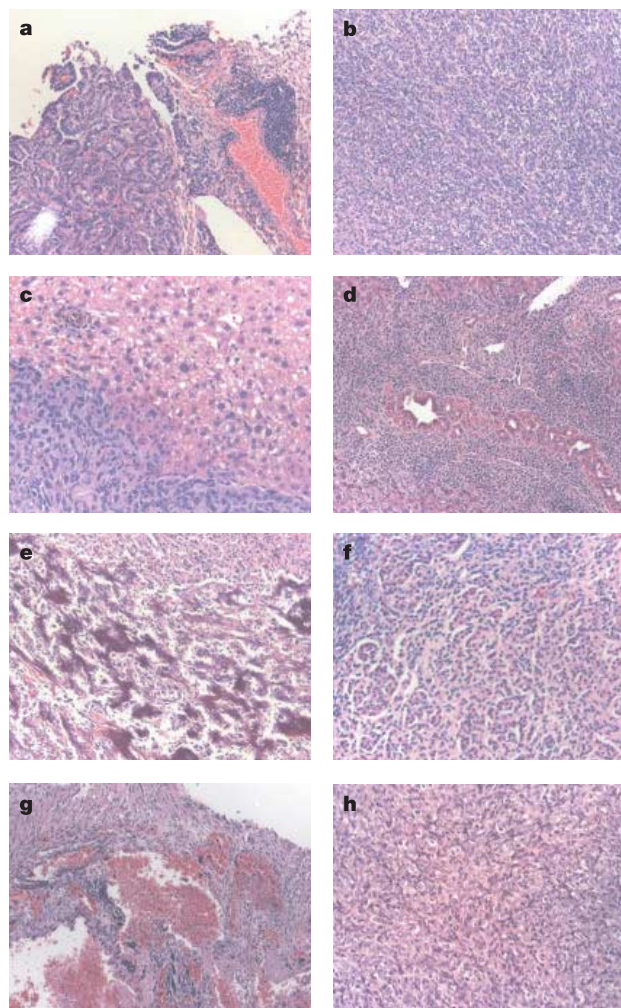


Figure 4 Development of multiple tumour types in p53^{+/–} *Fbxw7*^{+/–} mice. **a**, Lung tumour, adenocarcinoma; **b**, fibrosarcoma; **c**, liver tumour, hepatocarcinoma; **d**, liver tumour, cholangiocarcinoma; **e**, osteosarcoma; **f**, ovary, benign granulosa cell tumour; **g**, ovary, haemangiosarcoma; **h**, ovary, malignant granulosa cell tumour. Lung adenocarcinoma, hepatocarcinoma and haemangiosarcoma were also found in the irradiated *Fbxw7*^{+/–} mice (data not shown).

Western blotting

Total protein extracts were prepared from MEFs with RIPA lysis buffer. For western blots, 50 µg of protein extracts per lane were subjected to electrophoresis, transferred to poly(vinylidene difluoride) membranes (Millipore) and immunoblotted with anti-Notch4 (H-225; Santa Cruz), Notch1 (C-20; Santa Cruz) and anti-cyclin E (M-20; Santa Cruz), presenilin-1 (N-19; Santa Cruz), c-Jun (H-79; Santa Cruz), p53 (CM5; Novocastra Laboratories) and Aurora-A (BD Transduction Laboratories) antibodies; as control, the same membranes were stripped and immunoblotted again with anti-β-actin antibody (AC-15; Sigma). The membranes were washed and treated with rat anti-specific IgG^κ-chain secondary antibody conjugated to horseradish peroxidase (Amersham Pharmacia). The antigen-antibody reactions were revealed by using an enhanced chemiluminescence assay (ECL; Amersham Pharmacia) and exposed to enhanced chemiluminescence film.

Statistical analysis

Difference in frequency of LOH between p53^{+/-} and p53^{-/-} was assessed by genome-wide permutation testing. The Kaplan-Meier method was used to compare the survival time after irradiation between different groups of genotypes of mice with the use of the SPSS statistical package.

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Rubisco without the Calvin cycle improves the carbon efficiency of developing green seeds

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Efficient storage of carbon in seeds is crucial to plant fitness and to agricultural productivity. Oil is a major reserve material in most seeds¹, and these oils provide the largest source of renewable reduced carbon chains available from nature. However, the conversion of carbohydrate to oil through glycolysis results in the loss of one-third of the carbon as CO₂. Here we show that, in developing embryos of *Brassica napus* L. (oilseed rape), Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) acts without the Calvin cycle² and in a previously undescribed metabolic context to increase the efficiency of carbon use during the formation of oil. In comparison with glycolysis, the metabolic conversion we describe provides 20% more acetyl-CoA for fatty-acid synthesis and results in 40% less loss of carbon as CO₂. Our conclusions are based on measurements of mass balance, enzyme activity and stable isotope labelling, as well as an analysis of elementary flux modes.

During embryogenesis, seeds receive carbon precursors from the mother plant for the synthesis of storage products. In oilseeds such as *B. napus*, the dominant metabolic flux is the conversion of sugars into triacylglycerols³, resulting in more than 60% of the carbon being stored as oil (about 45% of dry weight). In described pathways for plant oil synthesis, sucrose is converted into pyruvate through glycolysis, which is then transformed into acetyl-CoA, the precursor of fatty-acid biosynthesis⁴. This conversion of sugars to oil entails the loss of one carbon as CO₂ at the pyruvate dehydrogenase (PDH) reaction for each two-carbon acetyl-CoA unit produced for oil synthesis (Fig. 1b). This loss of carbon seems to make oil storage less advantageous for seeds and raises the question why so many plant species use oil as their primary reserve in seeds and whether there is a more efficient way of transforming carbohydrates to oil. Because many seeds are green and have photosystems, even the decreased amount of light that penetrates the fruit wall⁵ can provide reductant and ATP that may expand the range of pathways available for storage product synthesis.

To measure the efficiency of carbon utilization during oilseed development, *B. napus* embryos were fed uniformly ¹⁴C-labelled carbon sources, and their conversion into oil, protein, carbo-

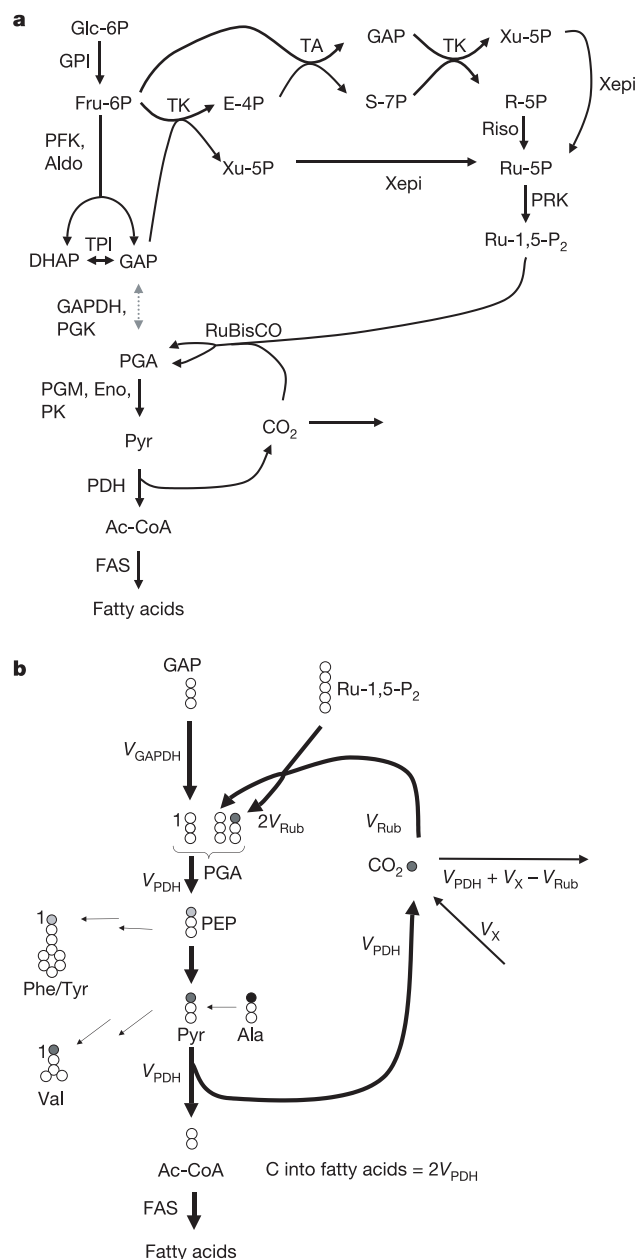


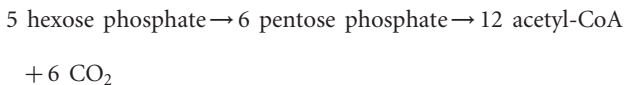
Figure 1 Metabolic transformation of sugars into fatty acids. **a**, Conversion of hexose phosphate to pentose phosphate through the non-oxidative steps of the pentose phosphate pathway and the subsequent formation of PGA by Rubisco bypasses the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase while recycling half of the CO₂ released by PDH. PGA is then further processed to pyruvate, acetyl-CoA and fatty acids. **b**, Part of **a** expanded to indicate carbon skeletons and to define relationships between V_{PDH} (flux through PDH complex); V_X (additional CO₂ production by the OPPP, the TCA, and so on); V_{Rub} (refixation by Rubisco). Metabolites: Ac-CoA, acetyl coenzyme-A; DHAP, dihydroxyacetone-3-phosphate; E4P, erythrose-4-phosphate; Fru-6P, fructose-6-phosphate; GAP, glyceraldehydes-3-phosphate; Glc-6P, glucose-6-phosphate; PGA, 3-phosphoglyceric acid; Pyr, pyruvate; R-5P, ribose-5-phosphate; Ru-1,5-P₂, ribulose-1,5-bisphosphate; Ru-5P, ribulose-5-phosphate; S-7P, sedoheptulose-7-phosphate; Xu-5P, xylulose-5-phosphate. Enzymes: Aldo, fructose bisphosphate aldolase; Eno, 2-phosphoglycerate enolase; Xepi, xylulose-5-phosphate epimerase; FAS, fatty-acid synthase; PGM, phosphoglyceromutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, phosphoglucose isomerase; Riso, ribose-5-phosphate isomerase; PDH, pyruvate dehydrogenase; PFK, phosphofructokinase; PK, pyruvate kinase; PGK, phosphoglycerate kinase; PRK, phosphoribulokinase; TA, transaldolase; TK, transketolase; TPI, triose phosphate isomerase.

hydrates and CO₂ was determined. Table 1 shows the partitioning of carbon into different biomass fractions and the proportion of carbon liberated as CO₂. The measured ratio of carbon stored in oil to carbon liberated as CO₂ was close to 3:1, which was substantially higher than expected. The formation of acetyl-CoA from pyruvate by PDH results in a ratio of 2:1, and CO₂ production from additional metabolic activities (such as the oxidative pentose-phosphate pathway (OPPP)⁶ and the tricarboxylic acid (TCA) cycle³) would decrease the ratio further. Thus, considerably less CO₂ was produced than expected from the amount of oil formed.

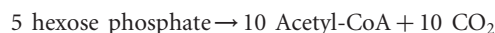
We considered whether the decreased CO₂ emission in light was due to its refixation by means of phosphoenolpyruvate carboxylase or pyruvate carboxylase. The product, oxaloacetate, could be converted into amino acids and stored in proteins or possibly secreted from the embryo (for example, in reduced form as malate). However, on the basis of the amino acid composition of *B. napus* embryos, recovery of CO₂ through oxaloacetate into seed protein can account for only about 4% of the CO₂ released by PDH (see Supplementary Information I). In addition, no export of malate or other such fixation products from embryos was detected (NMR data not shown). The observation of substantial Rubisco activity in *B. napus* seeds^{5,7} indicates that the reassimilation of CO₂ by the Calvin cycle might explain the increased efficiency of carbon use. The catalytic capacities of both Rubisco and phosphoribulokinase in developing *B. napus* seeds are sufficient to potentially fix all the CO₂ released by PDH⁷, and seed CO₂ levels⁸ saturate Rubisco carboxylase activity and prevent its oxygenase activity.

The results of labelling experiments show that CO₂ fixation by Rubisco is indeed active but that the Calvin cycle is not functional. Developing embryos were cultured in an atmosphere containing 2% ¹³CO₂—a concentration similar to the CO₂ levels that we and others⁵ measured in the gas-filled spaces, or locules, of developing siliques. Alternatively, alanine in the medium was replaced with [1-¹³C]alanine or [U-¹³C₃]alanine, which results in the release of ¹³CO₂ internally through the action of alanine aminotransferase and then PDH. In both experiments the distribution of ¹³C in amino acids and fatty acids shows that only the C1 carbon position of 3-phosphoglyceric acid (PGA) became labelled (Table 2). This is where CO₂ fixed by Rubisco is located; fatty acids, which are derived from C2 and C3 of PGA (Fig. 1b), were labelled to extremely low levels (Table 2). This labelling pattern is incompatible with flux through the Calvin cycle, in which the cyclic regeneration of ribulose-1,5-bisphosphate from PGA results in label from CO₂ being distributed into all carbon positions of the cycle's intermediates (including PGA)². We therefore concluded that although Rubisco is active in fixing CO₂ by the usual carboxylation reaction, it is not operating as part of the Calvin cycle but in a different context.

On the basis of the above results we conclude that Rubisco operates as part of a previously undescribed metabolic route between carbohydrate and oil (Fig. 1a). This involves three stages: first, the conversion of hexose phosphates to ribulose-1,5-bisphosphate by the non-oxidative reactions of the OPPP together with phosphoribulokinase; second, the conversion of ribulose-1,5-bisphosphate and CO₂ (most of which is produced by PDH³) to PGA by Rubisco; and third, the metabolism of PGA to pyruvate and thence to fatty acids (Fig. 1a). The net carbon stoichiometry of this conversion (for details see Supplementary Information II) is



By contrast, the conversion of the same amount of hexose phosphates through glycolysis is



Thus, Rubisco, together with the non-oxidative enzymes of the

Table 1 Observed and expected biomass production and CO₂ release by *B. napus* embryos in culture

Metabolic sink	Percentage of carbon observed	Percentage of carbon expected by conventional pathways
Oil	49.7 ± 2.8	<44.9*
Protein, starch, cell wall, etc.	32.7 ± 2.3	32.7
CO ₂ released	17.6 ± 2.2	>22.4*
Ratio of carbon in oil to CO ₂ released	2.9 ± 0.3	<2.0

Aseptically isolated embryos in the early phase of storage accumulation were grown in liquid culture for 3 days at a light intensity of 50 μmol m⁻² s⁻¹, with glucose, sucrose, glutamate and alanine as ¹⁴C-labelled substrates. Standard deviations are given for *n* = 5 experiments.

* Assuming that CO₂ is produced only for oil synthesis (PDH reaction), the carbon fractions of oil and CO₂ are expected to be in the ratio 2:1. This value is a minimum because, in addition to PDH, the OPPP and the TCA cycle produce CO₂ (see the text).

pentose phosphate pathway, allows the conversion of carbohydrate into 20% more acetyl-CoA and therefore oil than does glycolysis, with 40% less carbon lost as CO₂. This metabolic route is consistent with the mass balance data shown in Table 1, which, given the absence of substantial other CO₂ refixing mechanisms, are incompatible with the glycolytic route. Although Rubisco has a central function in the metabolic route that we describe, net fixation of CO₂ does not occur because the CO₂ fixed by Rubisco is subsequently released by PDH. The increase in carbon economy is therefore achieved only through the combined activity of Rubisco with the non-oxidative reactions of the pentose phosphate pathway.

We used two independent approaches to estimate how large a contribution this metabolic route makes to seed oil synthesis (at a light intensity of 50 μmol m⁻² s⁻¹).

First, the carbon balance of the embryo was used. The ratio of fatty-acid synthesis to CO₂ release can be formulated as (see Fig. 1b)

$$R = \frac{\text{carbon stored in fatty acids}}{\text{carbon released as CO}_2} = \frac{2V_{\text{PDH}}}{V_{\text{PDH}} + V_{\text{X}} - V_{\text{RuB}}} \quad (1)$$

where V_{PDH} and V_{RuB} are the fluxes through PDH and Rubisco respectively and V_{X} is the net CO₂ production due to the rest of metabolism (primarily the OPPP and the TCA cycle). This can be recast to allow V_{RuB} to be deduced from measurements of R and V_{X} :

$$V_{\text{RuB}} = V_{\text{X}} + V_{\text{PDH}} \left(1 - \frac{2}{R}\right) \quad (2)$$

We measured R to be 2.9 (Table 1) and determined that V_{X} is positive and small (see refs 3 and 5 and Supplementary Information). Accordingly, $V_{\text{RuB}} > 0.31V_{\text{PDH}}$. Because the great majority of PGA is converted to pyruvate and thence to acetyl-CoA⁹, the total rate of PGA production is very close to V_{PDH} . Because two PGA molecules are produced by the Rubisco carboxylase reaction, the rate of PGA production by Rubisco is $2V_{\text{RuB}}$. Thus, Rubisco is responsible for more than 62% of the total PGA production (46–75%, considering the range for R (Table 1)).

Second, we supplied [1-¹³C]Ala and [U-¹³C₃]Ala to developing embryos, which results in the release of ¹³CO₂ internally through

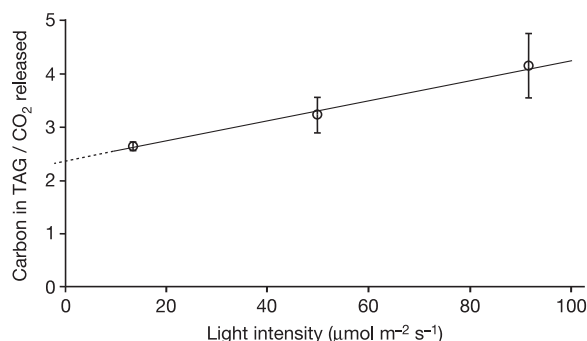


Figure 2 Light-dependent ratio of oil synthesis and CO₂ production. Embryos were grown at three different light levels for 24 h in closed culture flasks^{8,9}. After 24 h the CO₂ concentration inside the flasks was determined with an infrared gas analyser⁸, and the lipid content of embryo tissue was analysed by gas chromatography. Results are means ± standard error (*n* = 3). TAG, triacylglycerol.

the PDH reaction. The flux through Rubisco was estimated from the level of ¹³C in the C1 position of PGA-derived amino acids that arises from the direct incorporation of ¹³CO₂ by Rubisco. In the absence of flow round the Calvin cycle, the PGA that is produced by glycolysis from triose phosphate will be unlabelled, whereas half the PGA molecules produced by Rubisco are labelled in the C1 position (Fig. 1b). Thus, the labelling level in PGA is a function of the relative contributions of glycolytic flux and the flux through Rubisco, taking into account the enrichments in the precursors triose phosphate and ribulose-1,5-bisphosphate. The relationship (derived from metabolic and isotopic steady-state equations; see Supplementary Information III) is

$$\begin{aligned} \text{proportion of PGA from RuBisCO} &= \frac{2V_{\text{RuB}}}{2V_{\text{RuB}} + V_{\text{GAPDH}}} \\ &= \frac{2F_{\text{PGA}(1)}}{F_{\text{CO}_2}} \end{aligned} \quad (3)$$

where V_{RuB} and V_{GAPDH} are the fluxes through Rubisco and glyceraldehyde 3-phosphate dehydrogenase, respectively; F_{CO_2} and $F_{\text{PGA}(1)}$ are the fractional ¹³C enrichments in CO₂ within the embryos and in the C1 position of PGA, respectively. We determined $F_{\text{PGA}(1)}$ from Phe and Tyr carbon position 1 and we estimated F_{CO_2} from label in C1 of valine (Table 2, Fig. 1b). On the basis of the data in Table 2 (using $F_{\text{Phe}(1)}$ as well as $F_{\text{Tyr}(1)}$) and equation (3) we estimate that the flux through Rubisco is responsible for the production of 37–51% of PGA in the developing embryo. This estimate is a minimum because CO₂ produced by the OPPP is unlabelled, thus rendering the real value for F_{CO_2} smaller than estimated.

We next considered whether any other metabolic routes are possible for the conversion of hexose to fatty acids that could

Table 2 Refixation of ¹³CO₂ by Rubisco in cultured *B. napus* embryos

Measured metabolite	Corresponding biosynthetic precursor	¹³ CO ₂	Proffered labelled precursor [1- ¹³ C]Ala	[U- ¹³ C ₃]Ala
		Product fractional ¹³ C enrichment		
$F_{\text{Phe}(1)}$	$F_{\text{PGA}(1)}$	2.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
$F_{\text{Tyr}(1)}$	$F_{\text{PGA}(1)}$	3.0 ± 0.3	2.2 ± 0.3	
$F_{\text{Val}(2-5)}$	$F_{\text{PGA}(2-3)}$	0.2 ± 0.04	0.08 ± 0.05	
$F_{\text{oleic acid}(1-18)}$	$F_{\text{PGA}(2-3)}$	0.1 ± 0.01	0.1 ± 0.01	
$F_{\text{Val}(1)}$	$F_{\text{Pyr}(1)}$ (internal CO ₂)*	2.6 ± 0.1	8.6 ± 0.1	8.1 ± 0.1

¹³CO₂ was provided as external labelled substrate or was produced inside the developing embryo by metabolism of [1-¹³C]Ala or [U-¹³C₃]Ala. ¹³C incorporated into different biosynthetic products from 3-PGA (compare with Fig. 1b) is presented. Fractional ¹³C enrichment is denoted as $F_{\text{metabolite}(\text{carbon atoms})}$. Values (means ± s.d., *n* = 4) represent fractional ¹³C enrichment (above natural ¹³C abundance) in amino acids and fatty acids as determined by gas chromatography/mass spectrometry. Pyr, plastidic pyruvate. The biosynthetic precursor/product relations are taken from refs 6 and 9.

* For labelling with [1-¹³C]Ala and [U-¹³C₃]Ala the label in C1 of Val was assumed to represent the ¹³C enrichment in internal CO₂ (compare with Fig. 1b).

account for our observations. To do this we used elementary flux mode analysis^{10,11} applied to the enzymes of glycolysis, the OPPP, the Calvin cycle and fatty-acid synthesis (see Supplementary Information IV). This analysis showed that only the group of elementary flux modes that include the bypass of glycolysis by Rubisco can explain the observed increase in carbon conversion efficiency and labelling results. Three additional sets of flux modes were identified that describe the conventional glycolytic route, the Calvin cycle and the conversion of hexose phosphates to pentose phosphates through the oxidative decarboxylation of hexose with the subsequent formation of PGA through phosphoribulokinase and Rubisco. Linear combinations of the different flux modes showed that increased carbon efficiency is achieved only by increasing flux through Rubisco but comes at the cost of increasing requirement for external (photosynthetic) NADPH. The analysis also shows that with only a small fraction (less than 15%) of the NADPH or ATP required for the Calvin cycle, Rubisco in seeds can carry most of the metabolic flux to oil (see Supplementary Information IV). The flux distributions that are actually available to a developing seed will depend on the amount of light available for generating ATP and NADPH by photosynthetic electron transport. Indeed, as shown in Fig. 2, the ratio of carbon stored in oil to carbon liberated as CO₂ increases linearly with light intensity between 10 and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

In addition to demonstrating the operation of the new pathway in *B. napus* and the increased carbon efficiency that it confers, we also assessed the efficiency of carbon metabolism in developing non-green seeds. For two sunflower varieties developing in culture, the oil:CO₂ ratio ranged from 1.2 to 1.6 ($n = 3$ for each variety). This is similar to the ratio expected for the conventional glycolytic pathway to oil (taking into account some CO₂ release from respiration and the OPPP) and is in keeping with our prediction that the increased efficiency conferred by the new pathway requires light and photosystems to provide cofactors.

For green seeds, the presence of glycolysis, the OPPP, Rubisco and photosystems provides alternative metabolic routes that allow adaptations to different environments and thereby maximize the use of both the carbon provided by the mother plant and the light available to the embryo. The survival advantage to plants of the more efficient metabolic flux to carbon storage that we describe might explain why seeds of many species are green and contain substantial Rubisco activity during development despite the absence of sufficient light for the operation of the Calvin cycle. □

Methods

Isotopically labelled substrates

[U-¹⁴C]glucose, [U-¹⁴C]sucrose, [U-¹⁴C]glutamine and [U-¹⁴C]alanine were purchased from Amersham Biosciences. NaH¹⁴CO₃, NaH¹³CO₃, [1-¹³C]alanine and [U-¹³C₃]alanine were purchased from Sigma-Aldrich.

Embryo culture system

Embryos of *Brassica napus* L. (cv. Reston) collected early in the oil accumulation stage were cultured for 3 days in 5 ml liquid medium as described previously^{6,9}. Carbon sources were sucrose (80 mM), glucose (40 mM), glutamine (35 mM) and alanine (10 mM) at concentrations mimicking the endosperm liquid in which embryos develop *in planta*.

Measurement of the CO₂ balance for growing embryos

All organic carbon sources were uniformly labelled with ¹⁴C by adding [U-¹⁴C]sucrose, [U-¹⁴C]glucose, [U-¹⁴C]glutamine and [U-¹⁴C]alanine to the medium, resulting in a specific radioactivity of 1.42 mCi per mol of carbon. Culture flasks were sealed with sleeve stoppers containing two inlets. A 2% ¹⁴CO₂ atmosphere was created inside the flasks by

placing a glass vial into the flask containing 18.6 mg NaH¹⁴CO₃, and injecting HCl directly into the vial to release the ¹⁴CO₂.

Determination of ¹⁴CO₂ efflux

After culture, the flasks were flushed for 2 h with nitrogen at 45 ml min⁻¹ with exhaust gas bubbled through a 250-ml wash bottle containing 140 ml of 1 M KOH. The efficiency of the CO₂ trap for ¹⁴CO₂ recovery was on average 99.6% (s.d. = 3.3%). An aliquot (2.5 ml) of the trapping solution was counted by liquid scintillation.

Separation of ¹⁴C-labelled compounds

To extract lipids, ¹⁴C-labelled embryos were homogenized with a glass microgrinder at 4 °C in 1 ml iso-octane:isopropanol (2:1, v/v). The samples were centrifuged for 5 min at 5,000g and the supernatants were pipetted into glass test tubes (lipid fraction). Lipid extraction was repeated three times. To recover proteins, the pellet was extracted twice with 1 ml of 0.01 M sodium phosphate saline buffer (pH 7.4) containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.02% sodium azide and 0.0125% (w/v) SDS. After extraction of protein, a fibre/polysaccharide fraction was recovered by hydrolysis of the pellet in 0.5 ml of 67% aqueous sulphuric acid. ¹⁴C in each fraction was determined by liquid scintillation counting.

¹³CO₂ labelling

For labelling with stable isotope, embryos were grown for 3 days in a liquid medium as described above in a 2% CO₂ (v/v) atmosphere. CO₂ or alanine were replaced with ¹³CO₂, [1-¹³C]alanine or [U-¹³C₃]alanine, respectively. After growth, lipids and proteins were extracted, and fatty-acid methyl ester and amino acids were obtained as described previously⁹. Amino acids were analysed as *N,O*-t-butyldimethylsilyl derivatives and fatty acids as methyl esters by gas chromatograph/mass spectrometer as described earlier⁹. Mass spectra of Phe, Tyr, Val and oleic acid showed significant enrichment of ¹³C only in the m_1 peaks of each of the fragments observed. The apparent absence of molecules ¹³C-labelled at multiple positions (m_2 , m_3 , and so on) allowed the calculation of the fractional ¹³C enrichments in C1 of Phe, Tyr and Val as follows: $F_{\text{Phe}(1)} = m_{1,\text{Phe}(1-9)} - m_{1,\text{Phe}(2-9)}$; $F_{\text{Tyr}(1)} = m_{1,\text{Tyr}(1-9)} - m_{1,\text{Tyr}(2-9)}$; $F_{\text{Val}(1)} = m_{1,\text{Val}(1-5)} - m_{1,\text{Val}(2-5)}$; $F_{\text{PGA}(2-3)} = F_{\text{Val}(2-5)} = m_{1,\text{Val}(2-5)}$; $F_{\text{PGA}(2-5)} = F_{\text{oleic acid}(1-18)} = m_{1,\text{oleic acid}(1-18)}$ (fractional enrichment is shown as $F_{\text{metabolite}(\text{carbon numbers})}$, and mass peak m_x corrected for natural isotope abundance as $m_{x,\text{metabolite}(\text{carbon numbers})}$). Only in the case of feeding [U-¹³C₃]Ala did the mass spectra of Val and oleic acid reveal molecules to be labelled with ¹³C at multiple positions (significant abundance of m_2 , m_3 , and so on). In this case $F_{\text{Val}(1)}$ was calculated by subtracting the average ¹³C enrichment for fragments Val₁₋₅ and Val₂₋₅ from each other.

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Welcome competition

Every year a colleague of mine does a presentation to the new intake for the mass-media science fellows course at the American Association for the Advancement of Science. Her talk always includes a serious warning. Science writing is a treacherous, cut-throat world, she tells the prospective journalists, and there are few full-time jobs. "Are you sure you want to do this?" she asks, pausing before she adds: "Because I don't need the competition." In all honesty, this pitch could just as easily apply to scientists in general as to science writers — in either case, landing a full-time job straight after a PhD is difficult.

My friend's indoctrination talk would have been welcome at the inaugural meeting on scientific publishing in Bremen, Germany, last month. The meeting, organized by charitable foundation Bertelsmann Stiftung and the German Journalists' Federation, brought together scientists, policy-makers and journalists. But it did not offer specific advice on how to succeed in the field — just as scientists often receive little career advice at conferences, beyond the latest in scientific advances.

The turnout for the meeting, some 350 people, was a mix of established and aspiring science writers. Such a range gave a strong indication of the need for advice and information — especially as the economics of science-writing has caused a general shift from full-time jobs to freelance opportunities (see *Nature* 432, 418–419; 2004).

But there were some hopeful signs after the conference. Two German newspapers, *Die Zeit* and *Süddeutsche Zeitung*, are launching science sections this month. And career talks for scientists are now *de rigueur* at most US-based scientific conferences. I'm sure my colleague would agree that more of the same in Europe — for both traditional and off-the-bench scientific careers — would facilitate some welcome competition.

Paul Smaglik
Naturejobs editor



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